Diagnosis and Prognosis of Severe Dengue

Thesis

Diagnosis and prognosis of severe dengue

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ABSTRACT

Dengue has a broad spectrum of clinical symptoms, ranging from asymptomatic infection, undifferentiated fever and dengue fever (DF) to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF and DSS are the most severe forms of the disease, as they involve bleeding and hypovolemic shock and may lead to death if they are not diagnosed early and treated correctly. Every year, about 36 million cases of DF, of which about 2.1 million cases are DHF/DSS resulting in 21,000 deaths among children and young adults, are reported. Dengue not only has negative effects on public health worldwide but also poses significant economic burden. However, no licensed vaccines or (specific) anti-viral therapies for prevention or treatment of dengue are available. Thus, timely diagnosis and an accurate prognosis of severe dengue in the first few days of illness for clinical triage, treatment management, disease surveillance and research activities are vital and very helpful. We conducted three studies aiming to identify early markers of dengue, especially severe dengue and identify prognostic markers of severe dengue in Vietnamese children with clinically suspected dengue within the first few days of the illness. The results firstly showed that plasma collected within the first week of the illness could be used for dengue diagnosis as well as differentiation between primary and secondary dengue virus infections. Secondly, the research showed that plasma concentration of NS1 correlated with plasma viraemia and associated with DSS. NS1 concentrations in enrolment plasma of patients within the first four days of illness could be used for predicting DSS in patients with DENV-1 or DENV-2 infections. Thirdly, in the first three days of illness, we observed differences in clinical and haematological profiles between patients with DHF and those with DF or other febrile illnesses. Logistic models were then generated using the early clinical characteristics and/or haematological profiles to predict patients who will develop DHF. Our findings also showed that a greater virus burden leads to an augmented cascade of plasma concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα in DHF patients compared to DF patients. This thesis shows the results and also points out predictive models that may be used to prognosticate of severe dengue among patients with clinically suspected dengue who are admitted to hospital within the first few days of the illness.

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Abbrevations

aa	Amino acid
ADCC	Antibody-dependent cell-mediated cytotoxicity
°C	Degree Celcius
95%CI	95% confident interval
ADE	Antibody dependent enhancement
Ae. aegypti	*Aedes aegypti*
Ag	Antigen
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AUC	Area under curve
AUROC	Area under ROC curve
BHK-21	Baby hamster kidney cell line
BIC	Bayesian information criteria
C protein	Capsid protein
CD	Cluster of differentiation
cDNA	Complementary DNA
Ct	Cycle threshold
C6/36	a cell line derived from *Aedes albopictus*
DC	Dendritic cell
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue Haemorrhagic fever

DSS	Dengue shock syndrome
E protein	Envelope protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Link ImmunoSorbent Assay
HCMC	Ho Chi Minh City
HI	Hemaglutinin inhibition
HRP	Horseradish peroxidase
HTD	Hospital for Tropical Diseases
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Inter-quartile range (25^{th} - 75^{th} percentile)
JE	Japanese encephalitis
kb	Kilo-base pair
LLC-MK2	Rhesus monkey kidney cell line
mAb	Monoclonal Antibody
MAb	Mouse monoclonal antibody
MCP-1	Monocyte chemoattractant protein 1
MIG	Monokine induced by gamma interferon
µl	Microlitter
ml	Milliliter
ng	Nanogram
NK cell	Natural killer cell
NS	Nonstructural protein

OFI	Other fibrile illness
OPD	o-phenyleneediamine dihydrochloride
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
prM	Precursor Membrane
PRNT	Plaque reduction neutralization test
qRT-PCR	Quantitative RT-PCR
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
recE protein	Recombinant Envelope protein
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT-PCR	Reverse transcriptase polymerase chain reaction
TBEV	Tick-born encephalitis virus
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
U/L	Unit per litter
UC	Uncomplicated dengue
Vero	African green monkey kidney cell line
WBC	White blood cell
WHO	World Health Organization
WN	West Nile
YF	Yellow fever

1. Introduction

1.1. Introduction

The dengue virus (DENV) is considered to be one of the most important arboviruses in terms of its contributions to morbidity and mortality, and dengue has emerged as a major public health problem in tropical and subtropical countries around the world in recent decades. Dengue is caused by any one of four DENV serotypes (DENV-1 to -4) and is transmitted by *Aedes* mosquitoes. Dengue has a broad spectrum of clinical symptoms, ranging from asymptomatic infection, undifferentiated fever and dengue fever (DF) to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF and DSS are the most severe forms of the disease, as they involve bleeding and hypovolemic shock and may lead to death if they are not recognized early and treated correctly [1]. Although most dengue occurs in children under 15 years of age, the disease also occurs in adults. Dengue not only has negative effects on public health worldwide but also represents a significant economic burden including, but not be limited to, the costs of hospital care to patients and vector control activities [2, 3]. There are currently no licensed vaccines or anti-viral therapies for the prevention or treatment of dengue, although a number of these are in development [4, 5].

Thus, in order to reduce the high rates of morbidity and mortality caused by dengue, early, rapid, and accurate detection of DENV infection and clinical deterioration during the acute phase are vital. These are challenging problems, but they have the potential to help public health efforts to limit transmission, epidemic spread and reduce mortality.

1.2. Dengue epidemiology

1.2.1. Dengue epidemiology in the world

Dengue is the most rapidly spreading mosquito-borne viral disease in the world; in the last half century, dengue incidence has increased 30-fold and expanded to new countries (Figure 1-1 and Figure 1-2). There are approximately 3.6 billion people living in at-risk areas, with 70-500 million dengue infections per year. It is estimated that 36 million dengue fever cases and 2.1 million severe dengue cases occur annually in 124 countries, resulting in approximately 21,000 deaths [6].

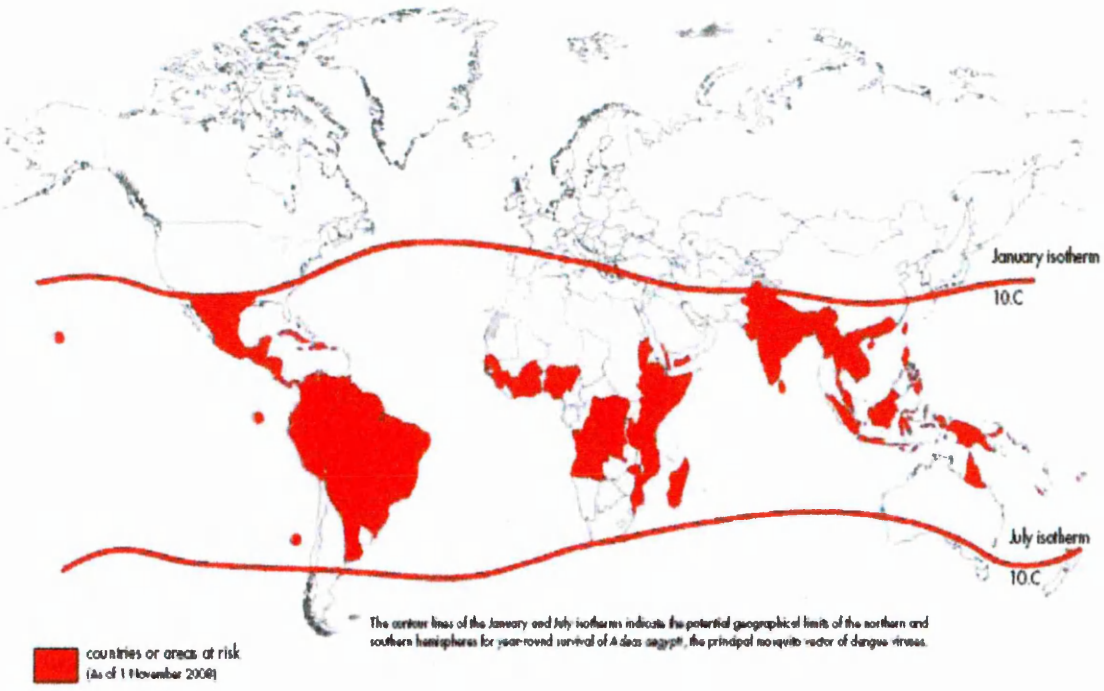


Figure 1-1: Countries/areas at risk of dengue transmission, 2008.

(Taken from [1]).

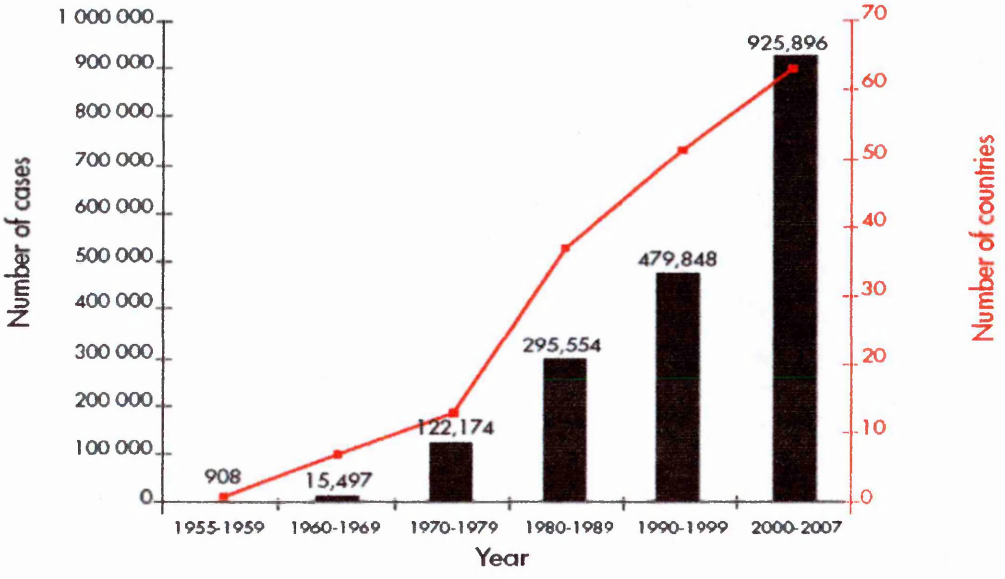


Figure 1-2: Average annual number of dengue cases reported globally and number of countries reporting to WHO by year, 1955 to 2007.

Taken from [1].

Two remarkable aspects of dengue epidemiology have emerged as global trends. Firstly, as described in a recent WHO document, dengue has rapidly spread into many new geographic areas and populations, and is now endemic in all WHO regions except the WHO European Region. More than 70% of the populations at risk for dengue worldwide reside in the WHO South-East Asia Region and Western Pacific Region, and these regions bear nearly 75% of the current global dengue burden. In the Region of the Americas, dengue has spread and the disease burden is characterized by cyclical outbreaks every 3-5 years. In the WHO African Region, there is evidence of dengue outbreaks which are increasing in size and frequency. The frequency and geographic range of outbreaks reported from the WHO Eastern Mediterranean Region have also increased since the past three decades after a reduction in dengue transmission in the 1940s due to vector control activities [1].

Secondly, although dengue has been reported predominantly among children, recent reports from Southeast Asia noted a shift in the age of dengue cases towards older age groups over the past three decades [7, 8]. This is important because numerous age-related differences in clinical manifestations have been shown among these older cases [9, 10]. This has led to calls for changes to clinical diagnosis, management and the dengue case classification [11, 12].

1.2.2. Dengue epidemiology in Vietnam

Dengue has been a public health problem in Vietnam for nearly a century, with tens of thousands of cases reported every year. Historically, a dengue-like illness was first recorded in Vietnam in 1913, when epidemics occurred in the north and the central provinces [13]. In 1929, the south of Vietnam experienced the first of many dengue fever epidemics. The first DHF outbreak in the country was identified in 1963 in the Mekong Delta region of southern Vietnam [14]. Between 1963 and 1995, Vietnam reported 1,518,808 DHF cases and 14,133 deaths [15]. Data from the dengue surveillance programme in southern Vietnam showed epidemic peaks of increasing magnitude occurring approximately every five years between 1975 and 1987, with a longer gap of 11 years preceding a large epidemic involving 119,429 DHF cases and 342 fatalities in 1998 [16]. Figure 1-3 illustrates important aspects of dengue epidemiology in Vietnam from 1960 to 2010.

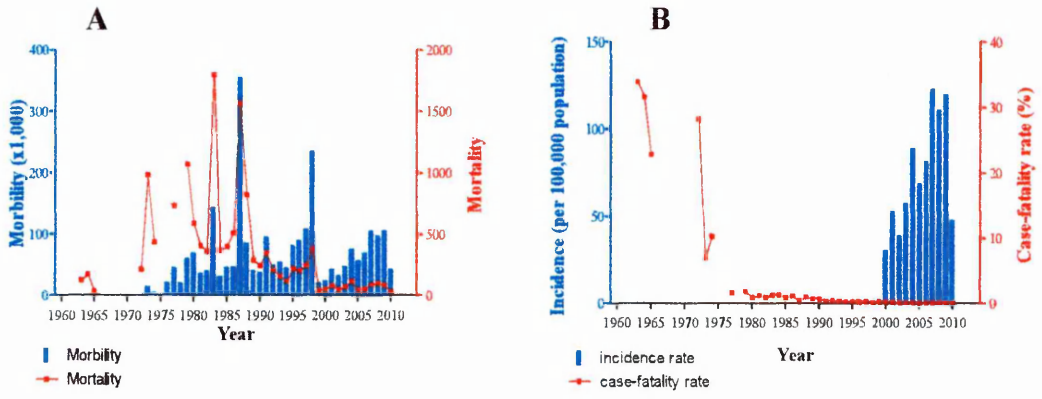


Figure 1-3: Vietnamese dengue cases and deaths officially reported from 1960-2010.

(Source: WHO, dengueNet *[17]*).

(A) Number of dengue (DF/DHF/DSS) cases and deaths in Vietnam. (B) Case-fatality rate and incidence rate in Vietnam. Incidence data was not available prior 2000.

In the southern 20 provinces of Vietnam from 1996 to 2008, the annual incidence of hospitalized dengue cases per 100,000 population peaked at 450 in 1998, then rose again to 263 in 2007 following a gradual increase over the previous decade (Figure 1-4). During the same period, the mortality rate progressively declined from approximately 0.3% in 1996, and leveled off at 0.1% in 2005 [18]. Similar trends were observed among children and adults at three referral hospitals in Ho Chi Minh City (Children Hospital No.1, No.2 and Hospital for Tropical Diseases, HCMC), with a rapid increase in dengue cases documented in adults. The annual dengue caseload steadily increased between 1996 and 2009, from around 8,000 to 12,000 cases per year in children and from less than 500 to more than 6,000 annual cases in adults (Figure 1-5). Dengue shock syndrome (DSS) accounted for 14.3% of paediatric and 1.6% of adult dengue cases; among all DSS cases, 1.6% resulted in death [19].

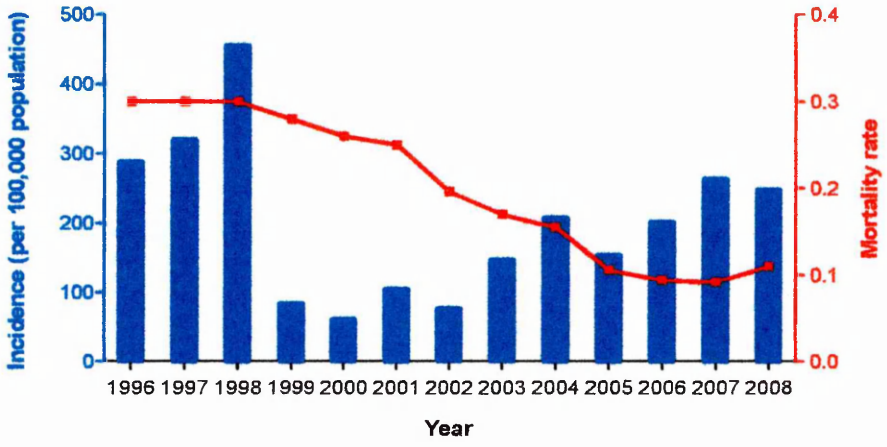


Figure 1-4: Annual incidence of hospitalized dengue cases per 100,000 population and mortality rate among dengue cases in southern 20 provinces of VN. (Source: Pasteur Institute HCMC, Vietnam [18])

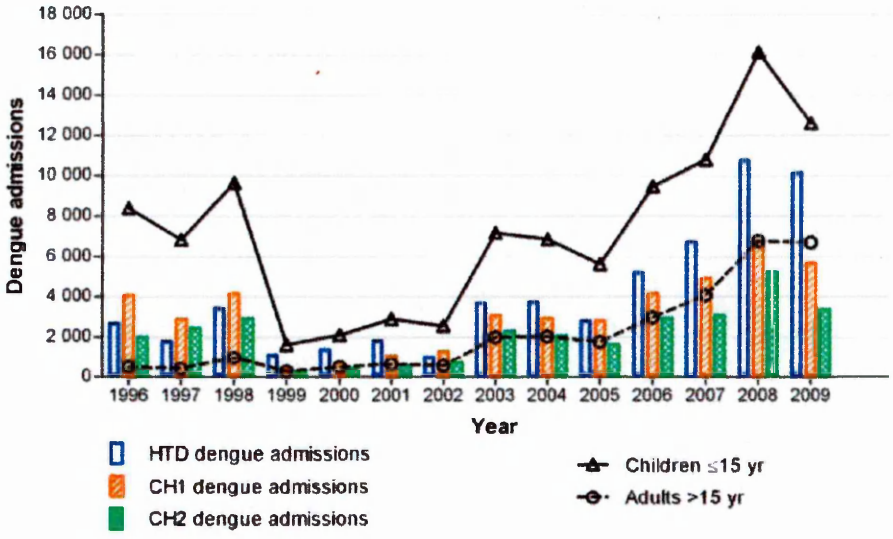


Figure 1-5: Temporal trends in dengue admissions in HCMC, Vietnam, 1996 – 2009. [19]

Bars show the number of inpatients with clinically diagnosed dengue each year at each of the major referral hospitals in HCMC (HTD: Hospital for Tropical Diseases, CH1: Children's Hospital No. 1 and CH2: Children's Hospital No. 2). Lines show the number of dengue cases in children aged 15 years or less (triangles) and in adults over 15 years old (circles), combined across the study sites.

All four serotypes of DENV circulate concurrently in the south of Vietnam, but a single serotype generally dominates over multiple dengue seasons. DENV-3 was dominant from 1997 to 1999, followed by DENV-4 from 2000 to 2002. Between 2002 and 2006, DENV-2 was the major serotype detected in hospitalized patients, with DENV-1, currently the most prevalent serotype, replacing it as the predominant lineage in 2007 [18].

1.3. The viruses

1.3.1. Dengue virus structure

DENV belongs to the *Flavivirus* genus of the *Flaviviridae* family, which contains almost 70 viruses, including those causing yellow fever and several encephalitides (e.g., Japanese, St. Louis, West Nile, and tick-borne) [20]. DENVs comprise four antigenically-related serotypes (DENV-1 to DENV-4), all of which are capable of causing disease. A mature dengue virion contains a single-stranded RNA genome surrounded by an icosahedral (20 identical equilateral triangular faces) nucleocapsid (core protein, C) and covered by an internal host-derived lipid bilayer membrane and an outer glycoprotein shell that consists of 180 copies each of an envelope (E) and membrane protein (M) [21, 22]. The complete virion is approximately 50nm in diameter [23].

The RNA genome is a single strand of positive polarity RNA with a length of approximately 11kb. It consists of two non-translated regions of 100bp and ~450bp at the 5' end and 3' end, respectively, and a single open reading frame. The open reading frame encodes for a polyprotein of approximately 3400aa. In the polyprotein, three structural proteins, C, prM – precursor membrane – or M and E, are synthesized first. Seven nonstructural proteins are then translated: NS1, NS2a and NS2b, NS3, NS4a and NS4b, NS5 with a function of NS3 – trypsin-like serine protease – responsible for processing of the polyprotein between NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 and possibly some processing of the capsid protein [24, 25]. Other junctions between C protein-prM, prM-E protein, E-NS1, and NS4A-NS4B are believed to be cleaved by host cell signalase in the lumen of the endoplasmic reticulum (ER) [22, 26, 27] (Figure 1-6).

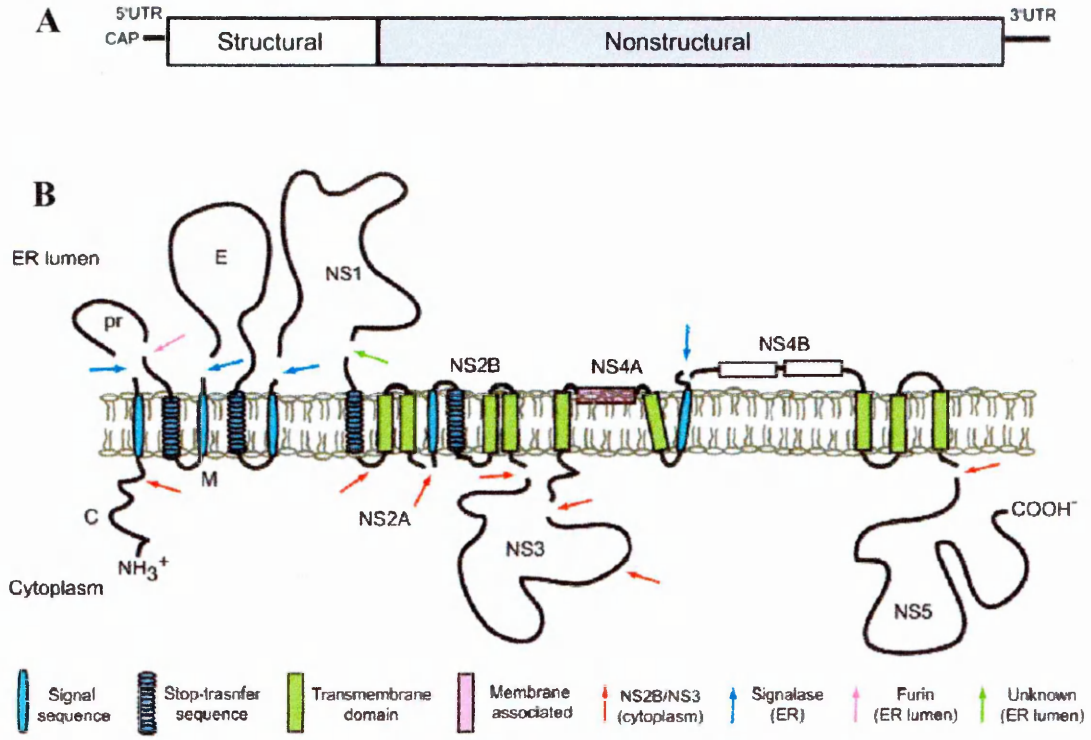


Figure 1-6: Schematic diagram of DENV genome and polyprotein processing.

A. The viral genome is a positive sense RNA of ~11kb in length. It is capped at the 5' end but lacks a poly-(A) at its 3' end. The structural proteins (open box) are encoded at the 5' one-third of the genome, followed by the nonstructural proteins (grey box). B. Membrane topology of the polyprotein. The viral RNA is translated as a polyprotein and processed by cellular and viral proteases (denoted by arrows). Source [22].

1.3.2. Protein function

The C protein is a small (9 to 12 kDa), highly positively charged protein that consists of ~120 amino acids, ~25% of which are lysine and arginine residues. This character probably enables the C protein to interact with virion RNA, functioning in the packaging of the viral genome and formation of the nucleocapsid core [28].

There are two forms of the M protein – prM, which is contained in immature intracellular virions, and M protein, which is contained in mature extracellular virions. The cleavage of the prM precursor (18 to 19 kDa) during viral maturation results in the formation of the 7 to 9 kDa M structural protein containing 75 amino acids. The

prM protein may function as a chaperone for the fold and assembly of the E protein [29, 30].

The E protein (54 – 60 kDa, ~500 amino acids) is the major protein component of the virion surface. It is involved in a number of biological activities, including receptor binding [31], hemagglutination of erythrocytes [32], and mediation of membrane fusion in acidic pH endosomes [21]. The E protein also induces neutralizing antibodies in the protective immune response [33-35]

The NS1 protein (42 to 50 kDa, ~350 amino acids) can be detected on the outer membranes of infected cells (dimer form), as well as in the extracellular milieu (hexamer form) [36, 37], and may play a role in viral replication [38]. The NS1 protein has been identified as a soluble complement-fixing antigen that can elicit antibodies and serve as a target for the immune response. Levels of secreted NS1 in plasma positively correlate with viral titers [39]. This makes it useful for diagnostic purposes, acting as a surrogate marker of viraemia [40] and early NS1 concentrations have been suggested to be useful as prognostic markers for DHF/DSS [39, 41].

The roles of the NS3 and NS5 proteins in viral replication are better understood. The NS3 protein (67 to 70 kDa, ~600 amino acids) plays helicase and protease functions that mediate post-translational cleavage of the DENV polyprotein; it also interacts with NS2B [42-45]. The NS5 protein (104 to 106 kDa, ~900 amino acids) is an RNA-dependent RNA polymerase that may also take part in the capping of viral RNA [46, 47]. The functions of the other small nonstructural proteins are not well understood. The NS2 consists of two proteins, NS2A (18-22 kDa, 218 amino acids) and NS2B (13-15 kDa, 130 amino acids); NS2A contains several possible transmembrane domains and participates in the processing of the C-terminal of NS1 [48], while NS2B is required for NS3 to function correctly [45]. NS4A (16.0-16.4 kDa, 150 amino acids) is critical to the rearrangement of intracellular membranes [49], while the function of NS4B (27- 28 kDa, 248-249 amino acids) has not yet been determined.

1.4. Transmission of dengue virus

DENVs are transmitted to humans through the bites of infected female *Aedes* mosquitoes. Mosquitoes generally acquire the virus while feeding on the blood of an infected person. After the incubation of the virus for 8-10 days, an infected mosquito is capable of transmitting the virus to other humans during probing and blood feeding.

Non-vector-borne transmission of dengue is also known to occur. Vertical transmission involves transplacental transmission from near-term pregnant women infected with DENV to the foetus [50]. Transfusion-related transmission [51], transplantation-related transmission (renal transplantation [52], bone marrow transplantation [53]) and needle-stick-mediated transmission have been shown to occur [54, 55] but are not common, as the infected blood or organs must be derived from a DENV-infected donor who is in the viraemic stage.

1.5. The vectors

Two major species of mosquitoes of the genus *Aedes, A. aegypti and A. albopictus,* are known to act as vectors in the transmission of DENV. Among these, *A. aegypti* is the principal vector for humans and is found in tropical and subtropical areas [56]. *A. albopictus* is not an urban vector of dengue, except in countries where *A. aegypti* is absent (parts of China, the Seychelles, Japan and Hawaii) [57]. In Asia, *A. aegypti* is regarded as the primary vector of DENV [58]. The eggs of *A. aegypti* are laid on the damp walls of clean water containers and can resist desiccation for several weeks or months. The lifespan of *A. aegypti* is 15 days on average, though it can live up for up to 174 days [59]. *A. aegypti* prefers to rest indoors and to feed on humans during the day; females often feed on several persons during the course of taking a single blood meal and, if infective, can transmit dengue virus to multiple persons in a short time, even if they only probe without taking blood [60].

After biting an infected human, dengue viruses enter and replicate firstly in cells of the mosquito's midgut. From there, the viruses gain access to different insect tissues. Viruses in the saliva of the infected mosquitoes are transmitted to humans during ingestion of blood meals. An infected female mosquito can transmit the virus for the rest of its life. Some evidences suggest the infected mosquito takes a longer

period of time to take a blood meal, thus enhancing the efficiency of transmission [61]. Moreover, transovarial transmission (via eggs), documented by Joshi and Gunther *et al.* [62, 63], allows the propagation of virus to the offspring during inter-epidemic periods without the participation of human or other vertebrate hosts, although there are still some doubts as to the overall significance of vertical transmission in dengue epidemiology.

1.6. Clinical management

Dengue has a wide clinical spectrum, including both severe and non-severe clinical manifestations. Most dengue patients recover without requiring hospitalization, while some infections may necessitate hospital care. The main principles of management of the disease are early detection of severe disease, careful supportive management, and adequate nursing care of patients. Appropriate management not only reduces the number of unnecessary hospital admissions, but also saves the lives of dengue patients (reduces the burden of disease).

1.6.1. Clinical diagnosis

Diagnosis of dengue cases is based on clinical symptoms, hematology and laboratory findings. The definitive diagnosis is made using laboratory techniques. The key to accurate diagnosis and treatment is prompt recognition and understanding of the clinical problems during different phases of the disease. In dengue, after an incubation period of 3-15 days with no signs or symptoms, illness progresses through three phases – febrile, critical and recovery [1] (Figure 1-7).

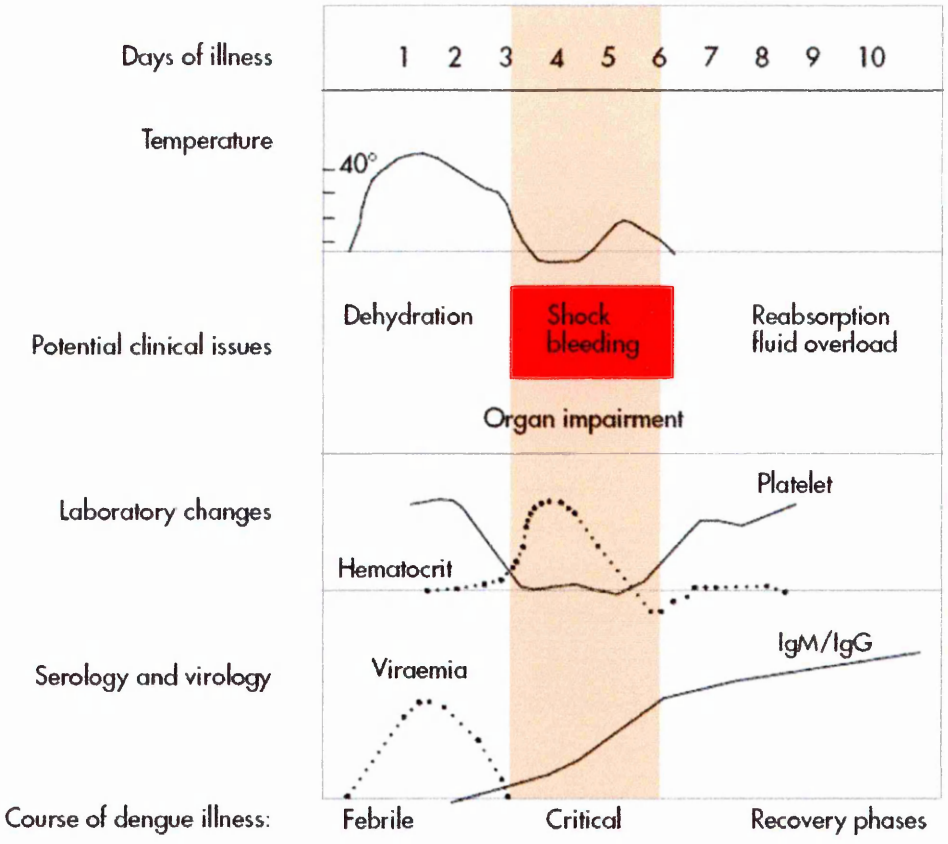


Figure 1-7: The course of dengue illness. (Taken from [1])

The acute febrile phase usually lasts 2-7 days. Early in this phase, patients typically develop a sudden high-grade fever and often experience facial flushing, skin erythema, body aches, myalgia (muscle pain), and headache. These may also be accompanied by anorexia, nausea and vomiting [64]. These signs and symptoms are not dengue specific and, thus, it can be difficult to clinically distinguish dengue from non-dengue febrile disease in the early febrile phase. Mild haemorrhagic manifestations such as petechiae and mucosal membrane bleeding (e.g. nose and gums) may also be seen [64, 65]. Vaginal bleeding and gastrointestinal bleeding may also occur during this early phase but are less common [64]. The earliest and most frequent abnormality shown in a full blood count in this stage is leucopenia; leucocyte counts have been described to be decreased in dengue relative to other febrile illnesses [66-68]. Elevated liver transaminases (Aspartate aminotransferase- AST and Alanine aminotransferase- ALT) also arise early in the course of the disease and have been documented to be higher in DHF cases [64, 69, 70].

The critical phase happens around the time of defervescence (temperature drops to and remains below 37.5-38^{0}C or less), usually on days 3-7 of illness; this phase may begin with an increase in capillary permeability concurrent with increasing haematocrit levels [71]. Progressive leucopenia [64] followed by a rapid decrease in platelet count usually precedes plasma leakage. The severity of plasma leakage is reflected by the degree of increase above the baseline haematocrit. Plasma leakage may cause pleural effusion and/or ascites visible by chest x-ray or abdominal ultrasound. At this point, patients who can compensate for capillary permeability will improve while those with increased capillary permeability may become worse (progress to shock) due to the loss of plasma volume. With prolonged shock, progressive organ impairment, metabolic acidosis and disseminated intravascular coagulation occur as the result of organ hypoperfusion. This in turn can lead to severe haemorrhage, which causes a decrease in haematocrit levels. Organ impairment such as severe hepatitis and encephalitis may also occur without signs of plasma leakage or shock. The period of clinically critical plasma leakage usually lasts for 24-72 hours.

The recovery phase begins with a gradual reabsorption of extravascular compartment fluid that occurs within 24-72 hours after the start of the critical phase. General health improves, the haematocrit stabilizes or may decrease and the white cell count starts to rise. However, the platelet count typically recovers later than the white cell count. Some patients may have a rash or may experience generalized itching during this phase. Some patients will have a so-called recovery rash – a florid macular rash affecting the limbs (Figure1-8).

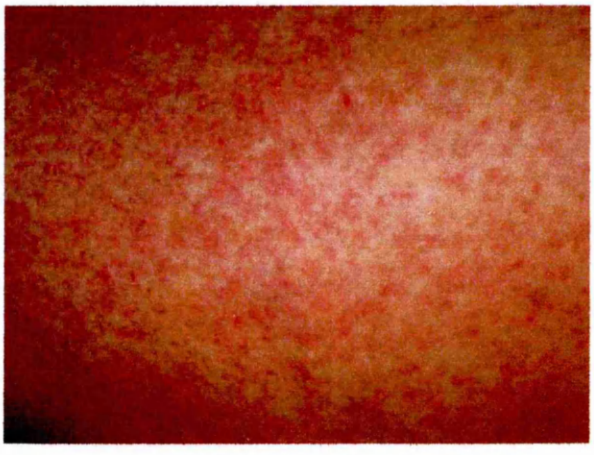


Figure 1-8: Recovery rash.

1.6.2. Case classification

The classification scheme suggested by the WHO in 1997 [72] divided symptomatic DENV infections into three groups: undifferentiated fever, dengue fever (DF) and dengue haemorrhagic fever (DHF). DHF was then sub-classified into four severity grades; among these, grades III and IV were defined as dengue shock syndrome (DSS). This classification scheme is now widely used. However, there have been many problems with the use of the 1997 WHO definitions because of changes noted in dengue epidemiology in recent years (such as the expansion of dengue to many new geographical regions and its changing age profile in some areas) [11, 12, 73, 74]. The difficulties in using these classifications and misclassification of many clinically severe dengue cases due to the strict criteria used to define DHF prompted requests for the WHO scheme to be reassessed. The WHO issued revised guidelines in 2009 [1] that seems to overcome most of these problems. In the new guidelines, DENV infections are now separated into two primary groups, dengue and severe dengue. The dengue group is then further divided into two subgroups – patients with warning signs and those without warning signs. Severe dengue shows one or more of the following manifestations: a/ plasma leakage that may lead to shock and/or fluid accumulation, with or without respiratory distress, b/ severe bleeding, and/or c/ severe organ impairment (hepatic damage, renal impairment, cardiomyopathy, encephalopathy or encephalitis). Criteria for the current classification scheme are shown in Figure 1-9.

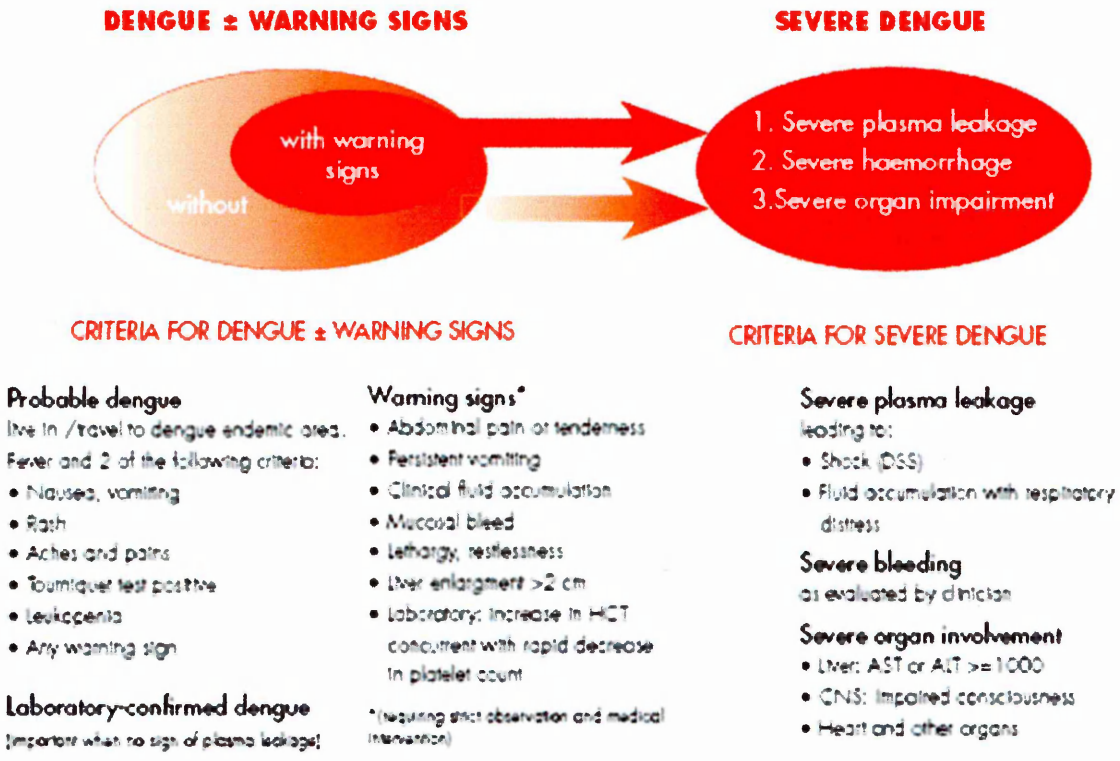


Figure 1-9: The WHO dengue case classification [1].

The WHO classifies dengue cases into two main groups - severe dengue and dengue. The large group of dengue has two subgroups – dengue with and without warning signs.

1.6.3. Differential diagnosis

Dengue fever can easily be confused with other infectious diseases [1]. Depending on the geographical origin of the patient and other etiologies, that may include malaria, leptospirosis and typhoid. Other diseases that mimic the early febrile phase of DENV infection are flu-like syndromes (influenza, measles, chikungunya, etc.), illnesses with a rash (rubella, measles, chikungunya, drug reactions), diarrhoeal diseases (enteroviruses) or illnesses with neurological manifestations (Meningo/encephalitis).

1.6.4. Treatment

There is no specific treatment currently available to combat DENV infection, although attempts to find an anti-viral therapy for dengue have been made. Case management is basically supportive and symptom-specific. With appropriate intensive supportive therapy, mortality may be reduced to less than 1% [75]. Maintenance of the circulating body fluid volume by encouragement of oral rehydration solution intake or adequate intravenous fluid replacement is the central feature of case management [1, 76-79]. Compounds or anti-viral drugs which block the viral entry pathway or virion replication have also been considered in an attempt to reduce viraemia and limit disease complications [22, 80]. Chloroquine which is used for malaria has been also taken into consideration for anti-DENV therapy; however, a randomized controlled trial of Chloroquine for treatment of dengue in Vietnamese adult showed that Chloroquine did not reduce duration of viraemia or NS1 antigenaemia [81]. Furthermore, several studies have been using corticosteroids for treating dengue but they have shown conflictive or inconclusive results [82].

1.6.5. Prevention and control

In general, three main strategies are considered integral to the prevention and control of dengue: control of mosquito vectors, development of vaccines and discovery of effective antiviral drugs. In the vector control aim, strategies include environmental changes (improved water supply, emptying or covering tanks, and underground reservoirs), personal protection (protective clothing, mats, and nets), biological control (fish, bacteria) and chemical control (insecticide) [3]. Problems with homotypic immunity (protective immune response against the same dengue serotype used for vaccine challenge), immune enhancement (which may lead to severe dengue) and lack of a suitable animal model for dengue disease [83] have hampered vaccine development. At the end of 2009, at least five dengue vaccines, including monovalent and tetravalent vaccines and using live-attenuated or chimeric viruses, were being investigated in phase I or II clinical trials [4, 5]. Lastly, in the field of antiviral drug development, efforts to find screening compounds are continually increasing. Most of the compounds currently under investigation target viral entry [84, 85], viral RNA polymerase/methyltransferase [86, 87], nucleotide

synthesis [88], viral helicase/NTPase [89], viral serine protease [90], R-glucosidases or kinases [91].

1.7. Current laboratory diagnosis

Diagnosis of DENV infection on the basis of clinical symptoms alone is not completely reliable, and should be confirmed by laboratory methods. The methods used to confirm DENV infection include detection of virus, viral nucleic acid, dengue antigens and virus-specific antibodies. Depending on the stage of the illness, an appropriate method or combination of all methods should be used (Figure 1-10). During the early stages of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis.

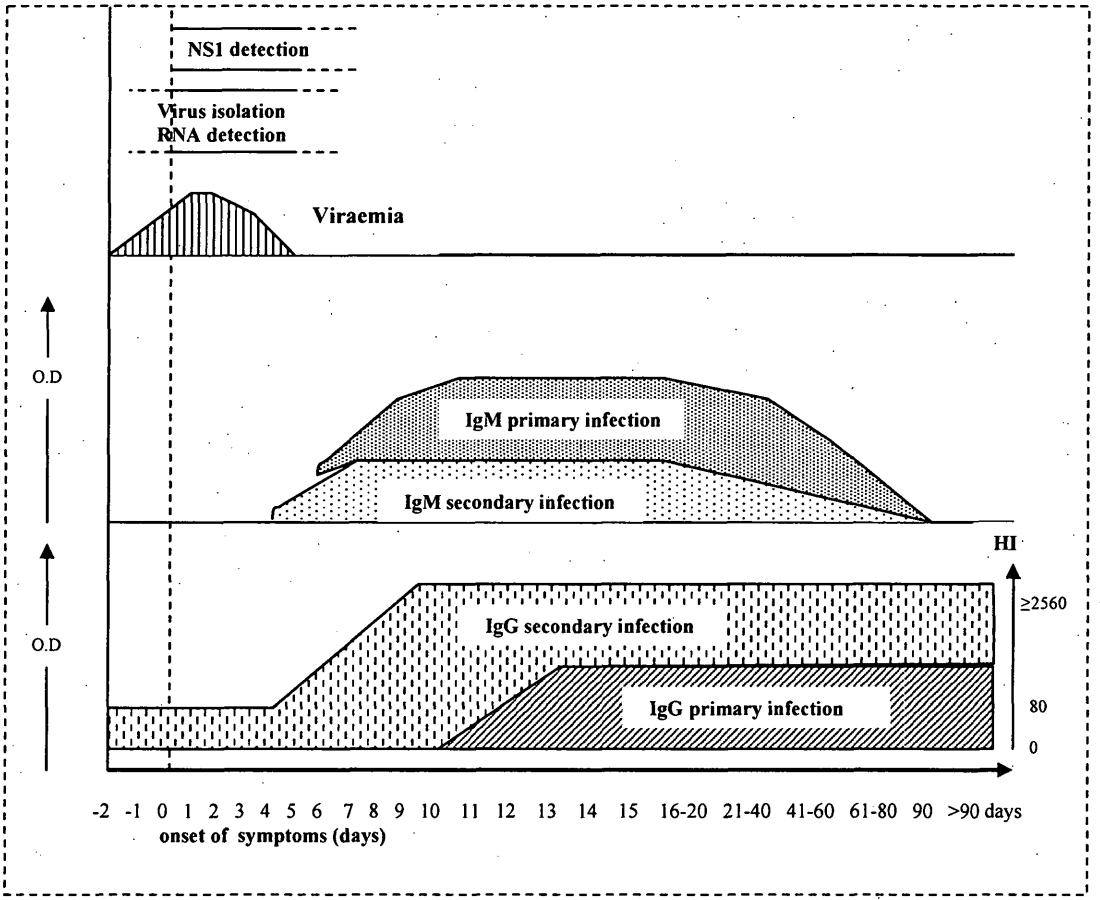


Figure 1-10: Approximate timelines of primary and secondary DENV infections and the diagnostic methods that can be used to detect DENV infection [1].

1.7.1. Virus isolation

DENV in serum, plasma, peripheral blood monocytes or tissue collected during an early stage of illness (usually before day 5) may be recovered by cell culture or inoculation in suckling mice or mosquitoes. Cell culture is the most widely used method for virus isolation, with C6/36 (a mosquito cell line) more efficient than mammalian cell lines (Vero, BHK-21 and LLC-MK2). The virus and serotype are identified by immunofluorescence assay using serotype-specific monoclonal antibodies. Although virus isolation is a gold standard for diagnosis of dengue and allows the infecting DENV serotype to be identified, this method is not appropriate for use in routine diagnostic laboratories because the procedure usually takes 1-2 weeks and requires specialized equiptment and reagents [1].

1.7.2. DENV nucleic acid detection

Several reverse-transcriptase polymerase chain reaction (RT-PCR) assays to detect DENV RNA have been developed since the early 1990s [92, 93]. Early methods allowed DENV serotypes to be detected and identified on the basis of their distinct amplicon sizes using specific primers. Later, real-time RT-PCR was developed and became an alternative method that used specific primers together with fluorescent probes for the detection of DENV RNA [94-96]. RT-PCR and real-time RT-PCR can be used to determine the presence of DENV infection in the early days of illness when the patient is still in the viraemic stage [1], and offer relative sensitivity compared with viral isolation and much more rapid time to detection (less than one day). It is possible that DENV RNA levels in serum or plasma could have prognostic value since Thai children with DHF have higher DENV RNA levels than children with DF [97]. However, these methods are expensive and necessitate specialized training as well as special equipment.

1.7.3. DENV NS1 antigen detection

Many studies have used NS1 as a target for the diagnosis of DENV infection. NS1 glycoprotein is secreted from mammalian cells and may last in circulating peripheral blood for up to 9 days of illness, even when DENV RNA cannot be detected by polymerase chain reaction (PCR) [98]. Several commercial kits (ELISA

or rapid tests) are now available for the detection of NS1 antigen. However, the sensitivity and specificity of these kits vary depending on the manufacturer [99, 100]. The sensitivity of NS1 testing also depends on the day of illness that the sample was collected [101-103]. Higher sensitivity has been documented within the first three days of illness and in primary cases [103-105]. Diagnostic methods based on NS1 antigen detection generally are not capable of identifying the infecting DENV serotype. However, a serotype-specific monoclonal antibody-based NS1 antigen-capture ELISA has recently been developed, and is suggested to be capable of detecting the infecting serotype and discriminating between primary and secondary DENV infections [106].

1.7.4. IgM/IgG antibody-capture enzyme-linked immunosorbent assay

IgM and/or IgG antibody-capture enzyme-linked immunosorbent assays (MAC-ELISA and/or GAC-ELISA) are cheap and easy methods used for diagnosis of DENV infection. MAC-ELISA has become an especially important tool in the routine diagnosis of dengue.

MAC-ELISA has good sensitivity and specificity but only when used on samples collected at 5 or more days after the onset of fever [1]. Cross-reactivity with other flaviviruses such as Japanese encephalitis, St. Louis encephalitis and yellow fever [107] is a limitation of the test, likely due to the use of whole cell virus antigen. This limitation can be overcome by using antigens from co-circulating flaviviruses as controls in MAC-ELISA. Different commercial kits (ELISA or rapid tests) are available, but sensitivities and specificities range from poor to acceptable or good; especially high rates of false positives were shown for sera obtained from patients with malaria, leptospirosis and past dengue infection [108].

GAC-ELISA is classically used for the detection of a current infection (if paired sera are collected within the correct timeframe). This assay uses the same antigens as MAC-ELISA and also shows low specificity within the flavivirus groups [109, 110]. An IgG antibody level or IgG avidity allows the identification of a case as resulting from a primary or secondary DENV infection [111-113].

IgM or IgG seroconversion in acute and convalescent paired sera is used to document acute infections [1]. In addition, the IgM/IgG ratio can be used to distinguish primary from secondary DENV infections [114-118]. However, the cut-off for the ratio is not well defined and varies between laboratories.

In general, tests with high sensitivity and specificity require more complex technologies and technical expertise, while rapid tests may compromise sensitivity and specificity for ease of performance and speed. Virus isolation and nucleic acid detection are more labour-intensive and costly but are also more specific than antibody detection using serological methods. Table 1-1 shows a comparison of the major dengue diagnostic methods.

Table 1-1: Summary of major dengue diagnostic tests used with information expected.

Diagnostic Test	Time of collection after onset symptoms	Time to results	Cost	Advantages	Limitations
Viral isolation and serotype identification	1-5 days	1-2 weeks	$$$$	- specific - possible to identify serotype	- need expertise and proper facilities - takes more than 1 week - cannot differentiate between primary and secondary infection - need acute samples
Nucleic acid detection	1-5 days	1 day	$$$	- possible to identify serotype - fast, so opportunity to impact patient management	- contamination - expensive - needs expertise and expensive laboratory equipment - cannot differentiate between primary and secondary infection - need acute samples
Antigen detection	1-6 days	1 day	$$	- easy to perform - fast, opportunity for early diagnosis may impact on patient treatment	- not as sensitive as virus isolation or RNA detection
IgM/IgG (paired sera)	Acute sera: 1-5 days; Convalescent: 7-15 days	7-15 days or more	$	- useful for confirmation of acute infection - least expensive -easy to perform - can distinguish between primary and secondary infection	- can miss cases because IgM levels may be low or undetectable in secondary infections - need two samples - delay in confirmation of diagnosis

Extracted from [1].

1.8. Pathogenesis and immunopathogenesis of dengue

Several hypotheses have been proposed to explain the pathogenesis of severe DENV infection. These below points are among the explanations for the aetiology of severe dengue.

1.8.1. The roles of serotypes, genotypes and viraemia

DENV serotype and viraemia levels have been found to be correlated with disease severity. For example, DENV-2 appears to be significantly associated with more severe dengue compared to DENV-1 [97, 119]. Genetic variations also exist within each serotype, resulting in genetically distinct genotypes. Several epidemiological observations and *in vitro* studies revealed a rapid replacement of Asian/American viruses by Asian 1 DENV-2 viruses [120] and that predominantly Asian genotypes of DENV-2 are more virulent than the American genotypes [121], with lineages of Asian origin known to produce higher viral titres [120, 122] and more strongly associated with DHF [123]. High levels of viraemia [97] and continued active viral replication or delay in the clearance of viraemia are also suggested to contribute to the pathogenesis of DHF [124-126].

1.8.2. The role of DENV NS1 antigen

The NS1 antigen is secreted from infected cells and plays a potential role in vascular leakage in severe dengue. High levels of NS1 are correlated with high viraemia levels [127] and associated with DHF [39, 41]. NS1 can also mediate complement activation, thus resulting in high levels of complement products (C5a, SC5b-9) in pleural fluids from DSS patients [41]. However, NS1 can protect DENV from complement-dependent neutralization *in vitro* because the binding of NS1 to C4 leads to inhibit complement activation [128]. Further, NS1 selectively binds to many types of epithelial cells, including those of the lungs and liver, but not intestine or brain endothelium [129]. The differential binding of soluble NS1 and subsequent recognition by anti-NS1 antibodies induce apoptosis in endothelial cells. This evidence has been suggested to explain the selective vascular leakage syndrome that occurs during severe secondary DENV infection [129].

1.8.3. Antibody responses

DENV-specific antibodies may have multiple functions in DENV infection. IgM and IgG antibodies against DENV provide immune protection through several mechanisms, including the blocking of cellular attachment, viral fusion [130] or increasing antibody-dependent cellular cytotoxicity (ADCC) [131, 132]. DENV infection induces a large repertoire of memory B cells that produce broadly cross-reactive antibodies specific for domain I/II of the E protein, while only a minority of memory B cells produce antibodies that bind to DIII, prM and nonstructural proteins [35, 133, 134]. Human IgG antibodies that bind to domain III of the E protein have greater neutralizing potency than those that bind to domain I or II (Appendix 1) while human antibodies that bind to prM don't neutralize infections but potently induce ADE *in vitro* [134]. Furthermore, acute phase sera from patients with DHF or DSS contain significantly higher levels of anti-DENV antibodies (IgA, IgG1, IgG4 and IgE) than those from DF and non-dengue patients [135-137]. In primary infections, IgM to DENV is the first immunoglobulin isotype to appear at high levels that greatly exceed IgG levels for 2 to 4 weeks. In secondary infections, IgM to DENV appears late at low levels during the febrile phase of illness, whereas high levels of IgG are detectable even in the acute phase.

Notably, infection with one serotype of DENV does not generate protective immunity to other serotypes, but it may result in increased severity from future heterotypic infections. During secondary infections, pre-existing antibodies at subneutralizing titer or non-neutralizing antibodies form complexes with the dengue virus particles and facilitate the infection of large numbers of Fcγ receptor-bearing cells [138]. This process is called antibody dependent enhancement (ADE). DENV-specific antibodies also exist in newborn infants [139]; when these antibody levels wane, DHF may occur during the first infection, possibly as a result of ADE [140].

1.8.4. Cell-mediated immunity

Many cell types are involved in the pathogenesis of dengue. Monocytes and macrophages have been considered as the primary target cells of the infection [141-145]. In addition, Langerhans cells or immature dendritic cells were found to be the cells that were most permissive of dengue infection, and were suggested as possible

early targets of infection [144, 146]; DENV infection stimulates the maturation of these cells and cytokine production. Furthermore, autopsy studies have suggested that hepatocytes may act as target cells for DENV [147, 148]. Infection causes liver injury, which leads to the elevation of serum levels of liver enzymes (AST and ALT) early in the course of disease; these are especially pronounced in DHF [64, 69, 70].

During acute infection, DENV-infected patients are usually leucopenic (low total cell counts), as characterized by a reduction of neutrophils and monocytes [64]. A recent gene expression case-control study found 21 genes relating to neutrophil activation or degranulation were expressed abundantly in patients who developed DSS [149]. Natural killer (NK) cells are also activated early in the acute phase [150-152] and play a role in lysing DENV-infected cells through direct cytotoxicity or via ADCC [153]. NK cells expressing CD69 are significantly increased in children during the acute phase of DHF compared to those with DF [151]. Besides, thrombocytopenia (low platelet count) is one of the key clinical manifestations in dengue and is an important cause of plasma leakage in severe dengue [154, 155]. Because platelets play an essential role in haemostasis, impairment of platelet function can increase the risk of vascular fragility, leading to haemorrhage.

Furthermore, the defence response is mediated by cells in the adaptive immune system. Excessive activation of T cells and B cells, especially of cross-reactive memory T cells, appears to augment the secretion of various cytokines during secondary infection [151]. Indeed, increased numbers of activated CD4+ and CD8+ T-cells and cytokine levels have been reported in DHF [156, 157]. The early T cell response to dengue epitopes has been defined in adults with secondary DENV infection, but the magnitude of this response was not found to correlate with disease severity [158]. Nonstructural proteins NS1, NS3 and the E protein were found to be recognized by HLA A11, A24 and B27-restricted CD8+ cytotoxic T cells [158, 159]. However, CD8+ T cells specific for $NS3_{133-142}$ were not detected before the commencement of haemoconcentration, thrombocytopenia or resolution of viraemia in Vietnamese children and thus it was suggested that these cells didn't play a crucial function in capillary leakage in DHF patients [160]. More studies are required to understand the contributions of T cell responses in the protection and pathogenesis.

1.8.5. Cytokine and chemokine responses

Monocytes/macrophages, B-cells, T cells and mast cells produce different cytokines and chemokines during the course of DENV infection, which may cause capillary leakage and in turn lead to shock in DHF cases. The types and levels of cytokines vary during the course of illness. Figure 1-11 summarizes the pattern of some cytokines relative to fever and viraemia during the course of the illness.

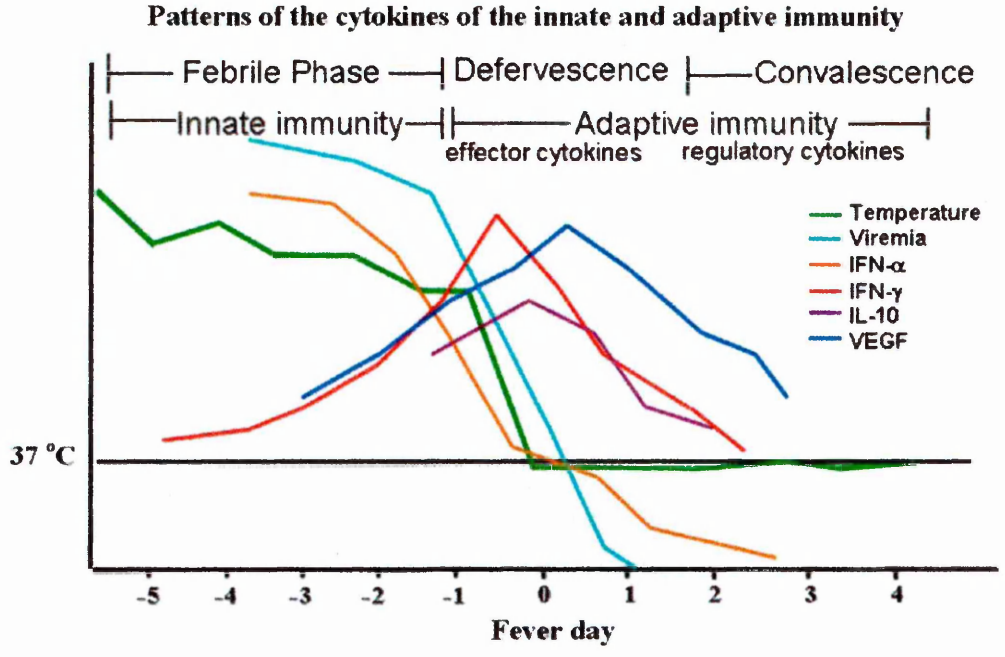


Figure 1-11: Temperature, virological and cytokine levels during the course of dengue virus infection.

Fever day 0 indicates the day of defervescence; fever days -1, -2, etc., indicate days prior to defervescence and fever days +1, +2, etc., indicate days after defervescence, respectively. Plasma leakage in DHF usually occurs around the time of defervescence, accompanied by a rapid decline in viraemia. Extracted from [161].

In the first three days of DENV infection, tumor necrosis factor-α (TNF-α), interleukin (IL)-2, IL-6 and interferon-gamma (IFN-γ) peak, whereas IL-10, IL-5 and IL-4 appear later [162]. High levels of IL-13 (an inhibitor of proinflammatory cytokines) and IL-18 (an inducer of IFN-γ) are also observed during the illness, with the highest levels shown in patients with DHF grade IV and lowest levels in patients with DF [163]. TNF-α, IL-10 and IL-8, cytokines with proinflammatory and vascular

permeability-enhancing activities, have been also reported to be elevated in DHF [164-168]. Moreover, multiplex cytokine profiles from adult dengue patients revealed that IL-1β, IFN-γ, IL-4, IL-6, IL-13, IL-7 and granulocyte-monocyte colony stimulating factor (GM-CSF) were increased in patients with severe dengue; in contrast, MIP-1β was higher in mild dengue [169]. Furthermore, the authors showed that MIP-1β and IFN-γ were independently associated with both dengue severity and disease outcome. Conflicting with these results, IL-2, IL-6 and IFN-γ have been shown to be equal in DF and DHF patients [170-172].

A study on gene transcription indirectly showed that multiple type I IFN–regulated genes were underexpressed in patients with DSS relative to those without DSS, suggesting important differences in the nature of the host immune responses [173]. In addition, complement, TLR and RIG-I signaling pathways, IFN-stimulated genes and cytokine/chemokines and their receptors were documented as the major features of the transcriptional signature in DSS [149, 174, 175].

In summary, the dengue literature contains multiple reports of increased plasma/serum cytokine concentrations during acute illness. However, because cytokine responses are common to all infections, it is very difficult to understand how sinigicant any of these cytokines is for the pathogenesis of capillary leakage. Furthermore, many studies on this topic come from different patient populations using different laboratory techniques making comparisions between studies difficult.

1.8.6. Host genetic influences

Although genetic mechanisms underlying the DENV-host interaction remain largely unknown, several host genes have been documented to contribute to the clinical outcome of dengue. For example, polymorphisms in HLA class 1 loci [176] and in five non-HLA genes (*IL-4*, *IL-1RA*, *MBL*, *VDR* and *FccRII*) were found to be associated with increased susceptibility to DHF children [177-179]. In contrast, variants of the vitamin D receptor (t allele at position 352) and FcγR IIA (R at position 131) were found to be associated with resistance to severe dengue [180]. Furthermore, a functional mutation in the promoter region of DC-SIGN was associated with susceptibility to mild dengue, but not DHF [181].

1.9. Biological markers of dengue severity

Studies of biological markers are critical because such knowledge may assist clinicians in identifying cases that will eventually develop into DHF/DSS, and may also contribute to the clinical management of disease. Many studies focused on identifying biological markers have suggested differences in markers present in the clinical samples of severe versus non-severe dengue cases. Such studies may provide early predictors that can be used to identify cases that are more likely to progress to DHF/DSS. Furthermore, such studies may assist investigators in finding novel approaches to combat plasma leakage and haemorrhage in severe dengue. Unfortunately, no remarkable biomarkers have yet been found. Table 1-2 summarizes the results of studies that found possible predictors with potential roles as biomarkers of dengue severity.

Table 1-2: Changes in biological markers in severity of dengue.

Study	Predictors	Comparison	Sample size	Age	Timing of sample	Note
Clinical laboratory markers						
Potts *et. al.*, 2010 [182]	WBC count, percent monocytes, platelet count, and hematocrit	DSS<non-DSS+OFI	648 OFI, 374 DF, 171 DHF I I/II, 37 DSS	6 months-15 years	Within 72 hours of illness	
Sosothikul *et. al.*, 2007 [183]	Platelet, von Willebrand factor antigen, tissue factor, soluble thrombomodulin	DHF<DF	20 DF, 22 DHF	Children	Febrile phase	
Pancharoen *et. al.*, 2002 [69]	Liver enzymes (AST and ALT)	DHF> DF	20 DF, 27 DHF, 57 DSS	Children	First 3 days after defervescence or shock	
Suvarna *et. al.*, 2009 [184]	Serum lipid profile (cholesterol, HDL, LDL)	DHF III/IV<DHF I/II<healthy controls	18 DF, 19 DHF I/II, 13 DSS, 50 non febrile controls	1 month-18 years	N/A	A case-control study matched by sex and age
Gorp *et. al.*, 2002 [185]			50 DSS, 20 DHF I/II, 20 healthy controls	children	up to 6 days of illness	

Study	Predictors	Comparison	Sample size	Age	Timing of sample	Note
Virological markers						
Wang *et. al.*, 2006 [124]	Viral level	DHF>DF	54 DF, 49 DHF	18-82 years	defervescence	All secondary DENV-2 infection, viral loads >5.7 log RNA copies/mL predicted DHF
Avirutnan *et. al.*, 2006 [41]	NS1 antigen	DHF>DF	49 DF, 44 DHF I, 44 DHF II, 26 DHFIII, 19 OFIs	2-15 years	Within 3 days before defervescence	
Libraty *et. al.*, 2002 [39]			14 DF, 16 DHF I/II, 2 DHF III	6 months-14 years	<=72 hours of illness	all DENV-2, NS1 level≥600ng/ml predicted DHF
Cytokines and chemokines						
Priyadarshini *et. al.*, 2010 [186]	IL-6, IL-8	DHF>DF	159 DF, 62 DHF	0 – >50 years (median age=24)	2-15 days of illness	
Bozza *et. al.*, 2008 [169]	IL-6, IFNγ, MIP-1β	Severe>mild (IFNγ, IL-6) Severe<mild (MIP-1β)	20 mild dengue, 39 severe dengue	15-73 years	3-10 days of illness	

Study	Predictors	Comparison	Sample size	Age	Timing of sample	Note
Dejnirattisai *et. al.*, 2008 [187]	CXCL9, 10 and 11	DHF>DF	14 DF, 26 DHF I/II, 10 DSS	N/A	Within 3 days before defervescence	
Chen *et. al.*, 2006 [188]	IL-6, IL-10, MIF	DHF non survivor>DHF survivor>DF	12 DF, 20 DHF, 17 healthy controls	7-79 years	1-18 days of illness	Cut-off values for fatal outcome: 68.6 pg/ml (IL-6) 267.8 pg/ml (IL-10) 54.7 ng/ml (MIF)
Nguyen *et. al.* , 2004 [189]	IL-6, IL-10, IFNγ, TNFα	DHF/DSS>control	85 DHF I/II, 22 DSS, 10 control infants	Infant <18 months	3-7 days of illness	
Azeredo *et. al.* , 2001 [190]	IL-10, IFNγ, TNFα	DHF, DF> healthy control	34 DF ,11 DHF, 15 healthy controls	10-82 years	Up to 7 days of illness	
Braga *et. al.* , 2001 [170]	IFNγ, TNFα	DF=DHF (IFNγ) DHF>DF (TNFα)	15 DF, 15 DHF	N/A	N/A	
Mustafa *et. al.* , 2001 [163]	IL-13, IL-18	DHF III, DHF IV> DF> healthy	24 DF, 10 DHF I, 24 DHF II, 13 DHF III, 13 DHF IV, 21 healthy controls	N/A	1 to >9 days of illness	

Study	Predictors	Comparison	Sample size	Age	Timing of sample	Note
Pacsa *et. al.*, 2000 [191]	IL-12	DF>DHF I>DHF II> DHF III, DHFIV, controls	16 DF, 10 DHF I, 24 DHF II, 13 DHF III, 13 DHF IV, 21 controls	N/A	1 to >9 days of illness	
Chaturvedi *et. al.*, 1999 [162]	IL-2, IL-6, IL-10, IFNγ, TNFα	DF>DHF (IFNγ, IL-2) DHF>DF (IL-6, IL-10) DF=DHF (TNFα)	35 DF, 12 DHF I, 35 DHF II, 21 DHF III, 14 DHF IV, 21 healthy controls	8 moths-55 years	1-18 days of illness	
Avirutnan *et. al.*, 1998 [165]	MCP-1	DSS>healthy control	6 DSS, 4 controls	N/A	N/A	
Soluble receptors						
Valero *et. al.* , 2008 [192]	sIL-2R	DSS>DHF	17 DF, 15 DHF, 12 healthy controls	Children and adult	5-9 days of illness	
Bethell *et. al.*, 1998 [193]	sTNFRII (p75)	DSS>DHF	184 non-shock (DHF), 71 pre-shock, 182 shock survivor, 6 fatal shock	children	Median days of illness: 4-5	sTNFR-75>55pg/ml predicted DSS

WBC: whole blood cell. OFI: other febrile illnesses. AST/ALT: Aspartate/Alanine aminotransferase. HDL/LDL: High-/Low-density lipoprotein. N/A: data not available.

1.10. Summary and directions of this thesis

In the last 50 years, dengue incidence has steadily increased across the world, expanded to new countries, and spread from urban to rural settings. However, the pathogenesis of dengue, especially that of severe dengue, is not well understood. It is likely that multiple mechanisms are involved in the pathogenesis of DENV infection, although their relative roles have not been determined. Furthermore, there are no licensed dengue vaccines or anti-viral therapies available for prevention or treatment of the disease.

Taken together, all of the issues described above have implications for the diagnosis and clinical management of dengue, as well as for the targeting of future vaccination strategies and therapeutic drugs. Moreover, in Viet Nam as well as other dengue endemic countries, it is not possible to admit all patients with clinical suspected dengue to hospital. Therefore, prompt diagnosis and classification are keys to case management and to reducing case fatality rates.

One of the major challenges of the disease is to identify cases in the early stages of illness, and to rapidly and accurately determine who will develop severe disease as the illness progresses. Predictions of disease progression are usually only made once patients have shown severe DHF symptoms [194]. Thus, biological markers that can be used to predict or serve as association factors with disease severity are urgently needed.

Finally, in the absence of animal models that can mimic human DHF, studies in dengue patients remain very important. However, limitations of these studies include small sample sizes, the often-delayed timing of sample collection and issues with the assignation of disease severity. In light of these problems, prospective studies with larger sample sizes, early recruitment and well-characterized patients with different degrees of severity are crucial in the effort to a better understand of the underlying mechanisms of the disease and to uncover early markers of dengue severity.

To contribute to these ends, the thesis is to identify some clinical or biological prognostic markers in a prospective study of a large group of children with dengue or

non-dengue recruited at an early stage of their illness. The following are three main objectives of the research:

1. Validate a serological test to confirm dengue and to classify primary and secondary infection;

2. Assess plasma NS1 levels in dengue patients and evaluate these for predicting disease severity;

3. Examine the clinical, haematological, virological and immunological characteristics of Vietnamese dengue patients and identify some biomarkers that can be used to predict the severity of dengue.

2. Materials & Methods

This chapter describes the general laboratory methods undertaken. Further relevant details are provided in subsequent chapters.

2.1. Definitions for terms used

Fever is defined as axillary body temperature above 37.5^{0}C.

Day one of illness is defined as the first day of the period when axillary body temperature is above 37.5^{0}C. Day of the illness (DOI) was self-reported by patient himself, parents or guardian.

Day of the defevescence is defined as the illness day on which the axillary body temperature first falls to ≤37.5^{0}C with no subsequent rise. Fefer day is the day which is compared to defervescence day. For example, fever day -1 is one day before defevescence; and fever day +1 is one day after defevescence.

2.2. Case definition

Dengue cases were defined according to the WHO 1997 guideline [72] as shown in table 2-1.

Table 2-1: Dengue case classification.

Classification	Essential Criteria (Fever plus X)
Probable DF	Two or more of the following: fever, headache, retro-orbital pain, myalgia arthralgia, rash, haemorrhagic manifestations, leukopaenia
DHF Grade I	Thrombocytopaenia (<100,000/mm^3) Evidence of induced, but not spontaneous, haemorrhagic manifestations Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings e.g. ascites
DHF Grade II	Thrombocytopaenia (<100,000/mm3) Evidence of spontaneous haemorrhagic manifestations Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings
DHF Grade III	Thrombocytopaenia (<100,000/mm3) Evidence of spontaneous haemorrhagic manifestations Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings Narrowed pulse pressure ≤20 mm/Hg Clinical signs of circulatory shock, e.g. refill time >2 secs, cold, clammy skin, cyanosis Rapid pulse
DHF Grade IV	Thrombocytopaenia (<100,000/mm3) Evidence of spontaneous haemorrhagic manifestations Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings e.g. ascites Pulse pressure=0 or undetectable blood pressure Clinical signs of circulatory shock, e.g. refill time >2 secs, cold, clammy skin, cyanosis Rapid pulse
Cannot classify	Don't meet the above criteria

2.3. Dengue Laboratory confirmed criteria

Together with the clinical syndromes of dengue, a diagnosis of "confirmed acute dengue" was obtained using an algorithm (Figure 2-1). The algorithm was based on 3 assays- a/ detection of DENV RNA in an acute plasma sample, b/ finding NS1 antigen in an acute plasma sample and c/ changes in DENV-reactive IgM or IgG levels in paired plasma samples. The detection of DENV RNA and NS1 antigen were performed on the acute sample (enrolment sample). The serological diagnosis (using IgM and IgG capture ELISAs) requires 2 samples at least 2 days apart and the 2nd sample collected on day six (or later) after the illness onset or on the early convalescent phase (it was usually the discharge sample).

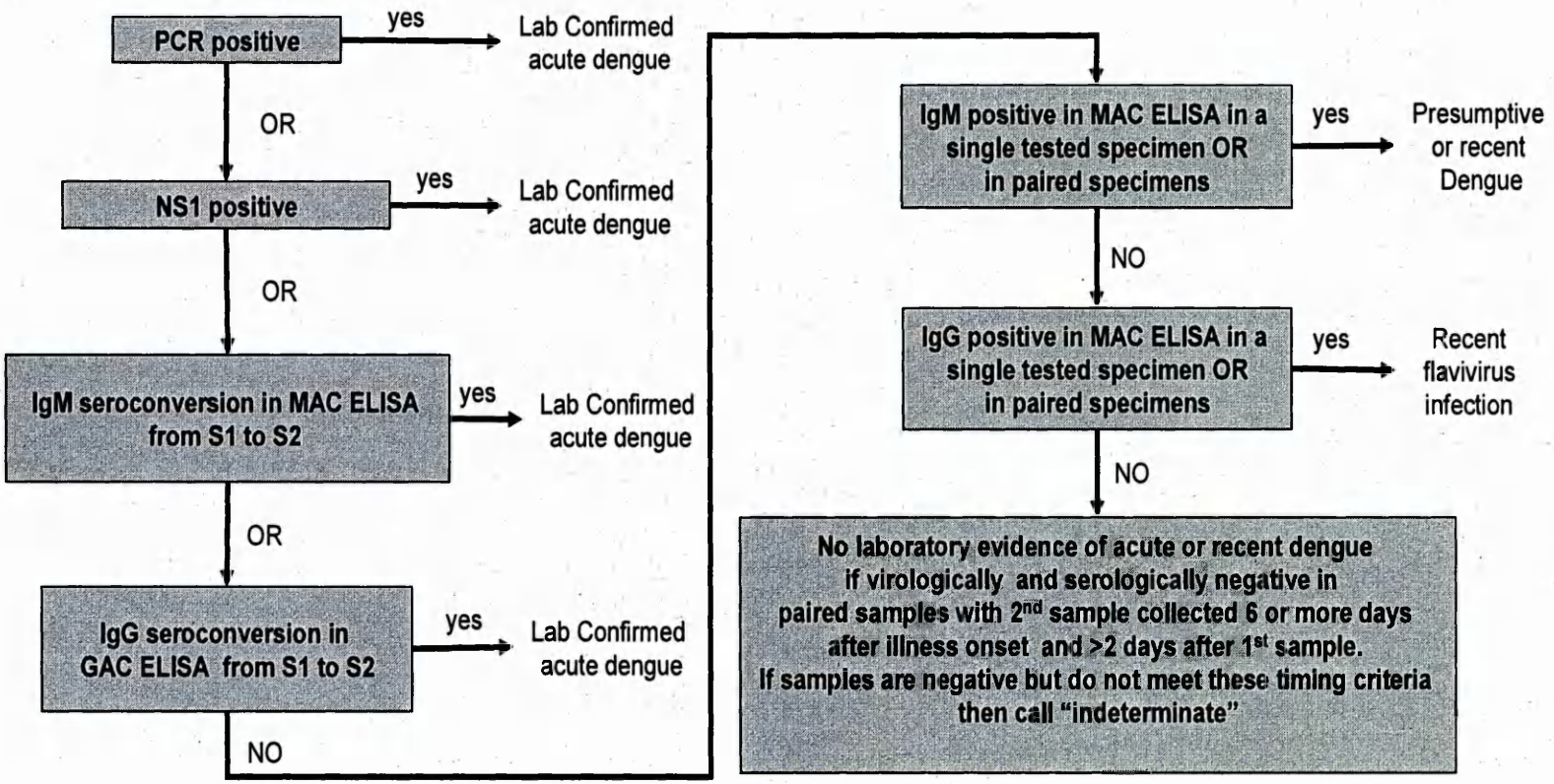


Figure 2-1: Dengue laboratory algorithm.

PCR and NS1 results are obtained from analyzing acute samples while IgM and IgG results are obtained from analyzing acute phase samples (S1) and early convalescent phase samples (S2). In the presence of a clinical syndrome that might be dengue, any of the first 4 tests is sufficient for a "laboratory confirmed" diagnosis.

2.4. Laboratory diagnosis

2.4.1. In house anti- DENV IgM and IgG capture ELISA assay

This is an in-house assay for detection of anti-Dengue/ anti-JEV IgM antibody. Antigens (culture supernatants from DENVs, JEV and mock infected C6/36 cells) and anti-DENV monoclonal antibodies were supplied by Venture Technologies (UNIMAS Research Park, University Malaysia Sarawak, Malaysia). Briefly, each well of ELISA plate (Maxisorp, Nunc Immuno plate, Cat# 442404) was coated with 100uL of anti-human IgM (DakoCytomation, Cat# A0425) or IgG (Sigma, Cat# I2136) diluted 1:2000 in 0.05M coating buffer (carbonate bicarbonate buffer, Sigma) then incubated overnight at 4°C. Plate was first washed three times with 300µL wash buffer (PBS containing 0.05% Tween 20) per well and blocked with 200µL PBS 3%BSA per well for at least two hours at room temperature. Plasma samples, positive and negative controls were diluted at 1:100 in sample diluent buffer (PBS containing 3%BSA). After a second washing of three times with 300µl wash buffer/ well, 100ul of diluted plasma and controls were added to triplicate wells and incubated for two hours at room temperature. After washing five times, 100µl of antigens (culture supernatants) was added to each well and then incubated at 4°C overnight. After another washing step (five times), a mix of mouse monoclonal antibodies to DENV were added and incubated for one hour, washed five times then anti-mouse Ig Horseradish Peroxidase (DAKO, Cat#P260) (1:2000 dilution) was added. After the final washing step, substrate o-phenylenediamine dihydrochloride (OPD) was added then incubated 30 minutes then the colour metric reaction was stopped by addition of 10% H_2SO_4. The Optical Density (OD) was read at 490nm.

Positive control was a mixture of plasma samples from acute dengue patients. Negative control was a mixture of plasma samples collected from 20 healthy Vietnamese donors. The cut-off value (CO) for positivity was defined as being 5 times higher than the average OD of negative control samples (ODn) after the subtraction of background OD (ODb). The sample ratio (Rs) was calculated by subtracting background OD from the test sample OD (ODs) then dividing by the assay cut-off. In summary, the formulas were calculated as below:

$$CO = 5x \text{ mean } (ODn - ODb)$$

Rs = (ODs – ODb)/ CO.

Result interpretation as below:

-If Rs<0.8, result was interpreted as negative.

-If Rs>1.2, result was interpreted as positive.

-If Rs from 0.8-1.2, result was interpreted as equivocal.

2.4.2. NS1 qualitative ELISA

Bio-Rad Dengue NS1 Ag Platelia ELISA kit is a one step sandwich – format microplate enzyme immunoassay for the qualitative or semi-quantitative detection of DENV-NS1 antigen in human plasma or serum. Tests were carried out according to the manufacturer's instructions. Briefly, 50µl of plasma or control sera (including one calibrator, one negative and one positive control sera) were incubated directly and simultaneously with 50µl of diluent and 100µl of diluted conjugate at 37°C for 90 minutes in microplate wells. The plate was washed six times with 1x washing solution (TRIS-NaCl – pH 7.4, 1% Tween 20), then immune-complexes were detected by using a color development reaction [160µl, mix of TMB Substrate Solution (citric acid and sodium acetate pH 4.0, H_2O_2 - 0.015%, and DMSO - 4%), and TMB Chromogen (0.25% of 3,3',5,5' tetramethylbenzidine)]. After 30 minutes of incubation in the dark at room temperature, the enzymatic reaction was stopped by adding 100µl of 1N sulfuric acid solution. The OD was read at dual 450/620 nm. The presence of NS1 antigen in each sample was determined by comparing the OD of the sample to the OD of the calibrator (Sample Ratio). The test would be validated if a/ mean of calibrator OD was greater than 0.2, b/ the negative control was lower than 0.4 time of the calibrator and c/ the positive control was greater than 1.5 times of the calibrator.

Result interpretation as below:

-If the Sample Ratio <0.5, result was interpreted as negative.

-If the Sample Ratio >1, result was interpreted as positive.

-If the Sample Ratio from 0.5-1, result was interpreted as equivocal.

2.4.3. RNA isolation

For screening and serotyping of DENV, viral RNA was isolated by using RNA extraction kits supplied by Qiagen (Qiagen, Cat#52906). For quantitative viraemia in serial samples from patients, Nuclisens easy MAG Extraction (BioMerieux, USA, Cat# 280131, 280132 and 280133) kits were used. Before extraction, a standard amount of Equine Arteritis Virus (EAV) was added into each plasma sample as an internal control for extraction and RT-PCR process. The procedure of RNA extraction was carried out following the manufacturer's instructions. Each specimen (100µl) was mixed with a lysis buffer containing a chaotropic agent which disrupts the three dimensional structure in macromolecules and inactivates any nucleases present in the specimen, thereby releasing the nucleic acids. Nucleic acids present in the lysate bound to carriers (that are beads in the Qiagen kit or are magnetic silica in the Neclisens easy MAG kit) under the high salt conditions. The carriers were then washed several times using wash buffers. Next, the nucleic acids were eluted from the carriers and concentrated in a specified volume (50µl) of the elution buffer. Finally, the concentrated nucleic acid solution was separated from the carriers by filter or magnetic field and then stored at minus 80°C until used.

2.4.4. Detecting and serotyping DENV

This method required two separate steps of PCR. RNA was first reverse-trancribed to cDNA and then the cDNA served as a template for real-time RT-PCR with appropriate DENV serotype-specific primers.

cDNA synthesis

cDNA was generated from viral RNA by reverse transcription using random hexamers. Random hexamers (Roche, Lewes, UK) were used at a working solution of 1.5µg/ml. A mixture of 8µl of RNA, 1µl of 1.5µg/µl random hexamers, 1µl of 10mM dNTPs, and 3µl of sterile distilled H_2O was incubated at 65°C for 5 minutes and then immediately chilled on ice for at least 1 minute. Another mixture of 4 µl of 5x First-strand buffer, 1µl of dithiothreitol (DTT, Invitrogen), 0.4µl of RNaseOUT (RNase inhibitor, Invitrogen) and 0.2µl of SuperScript III RT (reverse transcriptase,

Invitrogen) adjusted with H_2O up to 7µl was added to make up a reverse transcription reaction volume of 20µl. The content was mixed by gentle pipetting up and down then incubated at 25°C for 10 minutes. cDNA was then synthesized at 50°C for 60 minutes. The reaction was inactivated by heating at 75°C for 15 minutes.

Real-time RT-PCR for detecting and serotyping DENV

We used a DENV serotype-specific, fluorogenic real-time PCR assay to detect DENV RNA and to define DENV serotype in plasma samples. The dengue serotype-specific primers and probes were adapted from previously published sequences [195] and are summarized in Table 2-2. Hotstar Taq polymerase was used in all assays (Qiagen, USA). PCR reaction mixture is described in Table 2-3. PCR conditions were 95°C for 15 minutes then 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds then fluorescence was captured and then ending by 72°C for 30 seconds. A sample was validated if its PCR results showed EAV signals and was considered as having a specific serotype of DENV infection if any signal above the fluorescence background was seen in one of four serotype-specific PCRs.

Table 2-2: DENV Oligonucleotide primers, fluorescence-labeled probes

Serotype	Oligonucleotide sequences (5'-3')	Position
DENV-1 - Forward	ATCCATGCCCACCAYCAATG	9960 - 9980
DENV-1 - Reverse	CAGGGATCCACACCAYTGATC	10100 - 10121
DENV-2 - Forward	ACAAGTCGAACAACCTGGTCCAT	9938 - 9941
DENV-2 - Reverse	GCCGCACCATTGGTCTTCTC	10095 - 10115
DENV-3 - Forward	TTTCTGCTCCCACCACTTTCAT	9719 - 9741
DENV-3 - Reverse	TGGCGTTGGATGCYAGTCT	9915 - 9934
DENV-4 - Forward	GYGTGGTGAAGCCYCTRGAT	9587 - 9607
DENV-4 - Reverse	AGTGARCGGCCATCCTTCAT	9744 - 9764
EAV- Forward	CATCTCTTGCTTTGCTCCTTAG	1847 - 1869
EAV - Reverse	AGCCGCACCTTCACATTG	1962 - 1980
DENV-1-Probe	5' (FAM) TCAGTGTGGAATAGGGTTTGGATAGAGGAA-3' (TAMRA).	
DENV-2-Probe	5'(FAM) AGGGTGTGGATTCGAGAAAACCCATGG–3'(TAMRA)	
DENV-3-Probe	5' (FAM) AAGAAAGTTGGTAGTTCCCTGCAGACCCCA 3'(TAMRA)	
DENV-4-Probe	5' (FAM) ACTTCCCTCCTCTTYTTGAACGACATGGGA 3'(TAMRA)	
EAV - Probe	5'(Cy5) CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG 3'(BHQ)	

Y: C/T nucleotide, R: A/G nucleotide

R/F Primer: reverse / forward primer, respectively.

EAV: Equine Arteritis virus (causing systemic infection in equids)

Table 2-3: TaqMan RT-PCR mixture

Reagent	Working concentration	Volume (µl) / reaction			
		DENV-1	DENV-2	DENV-3	DENV-4
DENV F+R primer	10µM	1.5	1.5	1.5	1.5
EAV F+R primer	10µM	1.5	1.5	1.5	1.5
MgCl2	25mM	2.5	3.5	3.8	2.5
Buffer 10X		2.5	2.5	2.5	2.5
dNTP (Roche)	10mM	1	1	1	1
DENV Probe	2µM	2.5	1	3	2.5
EAV Probe	2µM	1.5	1.5	1.5	1.5
Hotstar Taq (Quiagen)	5U/µL	0.2	0.2	0.2	0.2
H_20 (Sigma)		7.8	8.3	6	7.8
cDNA		4	4	4	4

2.4.5. Quantification of DENV by one step RT-PCR (qRT-PCR)

The dengue serotype-specific primers and probes adapted from DENV serotyping PCR with some modifications are summarized in Table 2-4. We used LC480 RNA Master Hydrolysis Probes (Roche, Cat# 04991885001) which contain all essential reagents for PCR reaction. The PCR mixture is described in Table 2-5. PCR conditions included three steps. The first step was the reverse transcription step carried out at 61°C for 10 minutes. The second step was denaturation step carried out at 95°C for 02 minutes. And the amplification step was done with 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and then a fluorescence measurement was made. After all, the PCR products were brought to 37°C for 1 minute.

Table 2-4: DENV oligonucleotide primers, fluorescence-labeled probes using in the one-step RT-PCR

Serotype	Oligonucleotide sequences (5'-3')	Position
DENV-1 - Forward	ATCCATGCCCATCACCAAT	9865 - 9883
DENV-1 - Reverse	TGTGGGTTTTGTCCTCCATC	9945 - 9964
DENV-2 - Forward	TCCATACACGCCAAACATGAA	9859-9879
DENV-2 - Reverse	GGGATTTCCTCCCATGATTCC	9963-9983
DENV-3 - Forward	TTTCTGCTCCCACCACTTTC	9591 - 9610
DENV-3 - Reverse	CCATCCYGCTCCTTGAGA	9691 - 9708
DENV-4 - Forward	GYGTGGTGAAGCCYCTRGAT	9587 - 9607
DENV-4 - Reverse	AGTGARCGGCCATCCTTCAT	9744 - 9764
EAV- Forward	CATCTCTTGCTTTGCTCCTTAG	1847 - 1869
EAV - Reverse	AGCCGCACCTTCACATTG	1962 - 1980
DENV-1-Probe	5' (FAM) TCAGTGTGGAATAGGGTTTGGATAGAGGAA 3' (BHQ-1).	
DENV-2-Probe	5'FAM AGGGTGTGGATTCGAGAAAACCCATGG 3' BHQ1	
DENV-3-Probe	5' (Cyan500) AAGAAAGTTGGTAGTTCCCTGCAGACCCCA 3'(BHQ1)	
DENV-4-Probe	5' (Cyan500) ACTTCCCTCCTCTTYTTGAACGACATGGGA 3'(BHQ1)	
EAV - Probe	5'(Cy5) CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG 3'(BHQ)	

Table 2-5: One step RT-PCR mixture

Reagents	Working concentration (μM)	μl/reaction
DENV Primer	20	1.00
DENV Probe	10	0.28
EAV Primer	20	0.20
EAV Probe	10	0.08
Activator (Roche)		1.40
Enhancer (x20) (Roche)		1.00
Master mix (Roche)		7.40
H_20 (Roche)		2.64
RNA		6.00
Total		**20.00**

2.4.6. Cytokine detection by multiplex micro-bead immunoassay

The principle of this assay is a multiplex biometric immunoassay, in which many fluorescent-dyed microspheres are used to detect cytokines (in our assay, 10 kinds of microsphere beads). Each kind of microspheres is coupled with monoclonal antibodies specific for a desired target cytokine. Bound cytokines are then detected by biotinylated detection antibodies specific for different epitopes. Streptavidin-phycoerythrin is then added to bind to the biotinylated detection antibodies on the bead surface. In this study, kits (Bio-Plex Human Cytokine Assay, Bio-Rad Inc., USA) were used for cytokine measurement following the manufacturer's instructions. The kit includes concentrated human recombinant cytokines as standards and coupled beads for 10 cytokines measurement, such as: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα. Briefly, 50μl plasma samples were incubated with coupled beads. Complexes were washed twice, then incubated with biotinylated detection antibodies and, finally, labeled with streptavidin-phycoerythrin prior to assessing cytokine concentrations. Cytokine concentrations were determined using a multiplex array reader from Luminex Systems (Bio-Plex Workstation from Bio-Rad Laboratories) and the analyte was calculated using software provided by the manufacturer (Bio-Plex Manager Software). Standard ranges of cytokines that could be detected are shown in table 2-6.

Table 2-6: Detection ranges of cytokines (in pg/ml)

Description	IL-1b	IL-2	IL-4	IL-5	IL-6	IL-10	IL12-p70	IL-13	IFN-g	TNF-a
Minmum	0.23	0.84	0.14	1.5	1.23	0.96	0.2	1.19	0.34	0.14
Maximum	4,438	3,822	2,387	1,759	21,550	13,617	3,445	6,729	6,640	592

2.5. *Statistical analysis*

Data was analyzed with SPSS statistical package version 14.0.0 for Windows (SPSS, Inc, Chicago, III) or R 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria); and GraphPad Prism version 5 (GraphPad software Inc., USA) was used to plot graphs. Dichotomous measures were compared using the Chi-square test or the Fisher Exact test where appropriate. The Mann-Whitney U test was used for continuous variables. A two-tailed p value of below 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves, which were made by plotting the true-positive rate (sensitivity) versus the false-positive rate (1 – specificity), were used to evaluate the diagnostic performance of bio-markers at various cut-off points. Using standard formulas, the following performance measures were used to quantify the performance of the predictions from the selected models: the area under the ROC curve, sensitivity, and specificity, positive and negative predictive value of the cut-off value that represented the appropriate discrimination.

The accuracy of the proposed model was evaluated through two methods. The model was first assessed by using the original data set that generated the model (training data set) to derive apparent (by simple resubstitution) and optimism-corrected value. The optimism-corrected value was derived by using bootstrap method (with 100 times repeated) to assess potential overfitting of our models (including the model selection process) when using the same data set that had been used to generate the models [196]. And then, if another independent test data set was available, the performances of the models were calculated and compared.

3. Discrimination primary and secondary acute dengue virus infection

3.1. Introduction

Many tests to classify serological responses during acute DENV infection have been employed around the world. The HI test has been widely used and relies on the ability of antibodies to block the haemagglutinating activity of DENV, with high titres in the HI test highly suggestive of secondary infection [197]. Later, the ratio between IgM and IgG has been employed to classify primary and secondary immune status. This approach is based on the observation that acute phase sera from patients with primary infections have higher IgM/IgG ratios than those from patients with secondary infections [114-118]. Matheus et al. proposed an IgG avidity test for the differentiation of primary from secondary infection. This test is based on the changes in the avidity of IgG with the pathogen during the infectious episode, the avidity level of primary sera was always lower than that of the secondary sera [112, 113, 198]. An NS1 serotype-specific IgG ELISA for serological classification was first proposed by Shu et al. [106]. The assay is based on the fact that NS1 serotype-specific IgG antibody can't be detected in primary sera until day 9 of illness, so the NS1 serotype-specific IgG antibodies measured in acute sera must come from previous infections.

The HI test is still recommended by the WHO for discrimination between primary and secondary infection [1, 72]. However, the drawbacks of the HI test for routine laboratory diagnosis of dengue include 1/ using two samples that had to be collected at an appropriate time interval, 2/ requirement for chemical pretreatment to remove non-specific inhibitors and agglutinins and 3/ the high rate of cross-reactions with other flavivirus-specific antibodies [199]. Instead, the simpler immunoglobulin M (IgM) and G (IgG) antibody capture enzyme- linked immunosorbent assays (MAC and GAC ELISA) have become practical assays for the diagnosis of dengue [106, 200] because they are generally fast, high throughput and use relatively inexpensive reagents.

Antibody profiles elicited by primary and secondary DENV infections differ. Detectable anti- DENV IgM and IgG antibody generally become measurable within 4-6 days of fever onset. IgM wanes after 60-90 days [114] [201] but IgG remains measurable for many years after infection. In primary infections, IgM antibodies develop rapidly with high level while IgG appears slowly afterwards. In secondary

infections, high levels of IgG are detectable even in the acute phase whereas IgM levels are low or even absent [202]. One of the challenges in dengue serology is determining primary from secondary infection on the basis of ELISA results. In an ELISA assay developed in the AFRIMS (Armed Forces Research Institute of Medical Science) laboratory in Bangkok, Thailand, an optical density ratio of IgM:IgG of >1.8 in acute plasma classified a patient as having primary infection. The day of illness that the sample was collected was not factored into this classification system. Moreover, the ratio of 1.8 is valid only in this particular assay and cannot be exported to other IgM and IgG ELISA assays that use different reagents from the AFRIMS assay.

In this study, we aimed to validate a DENV IgM and IgG capture ELISA in the detection and differentiation of primary and secondary infection. In detail, the objectives of this chapter are:

1. To evaluate the in-house Hospital for Tropical Diseases DENV IgM ELISA test for sensitivity and specificity.

2. To generate a classification model based on IgM and IgG levels and day of illness at the time samples were collected to classify primary and secondary dengue cases.

3. To evaluate the classification model.

3.2. Methods

3.2.1. Reference panel for evaluation of in-house IgM assay

To evaluate the Hospital for Tropical Diseases (HTD) in-house anti-DENV IgM ELISA test for detection of IgM against DENV, a panel of reference serum samples was used. The panel was described in detail by Hunsperger *et. al.* [108] and was collected by a consortia supported by the United Nations International Children's Emergency Fund/United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Disease and the Pediatric Dengue Vaccine Initiative. All samples were coded to ensure that the person performing the assay was blinded to sample identity.

3.2.2. Samples for discrimination of primary and secondary responses using the in-house assays

To generate and evaluate a classification model for primary and secondary dengue using the in-house assays, we used plasma samples from dengue patients enrolled in prospective studies at the HTD and a private, primary level health care clinic located in HCMC. All patients admitted to HTD were in-patients, and the clinic treated out-patients. The samples were from patients aged above 4 years old, had fever within 72 hours of study enrolment and were laboratory confirmed acute dengue cases. Two venous blood specimens were drawn from each patient at the time of enrolment (acute phase) and again 3-6 days later (at the time of discharge- early convalescent phase). Acute dengue cases were defined by having seroconversion in DENV-reactive IgM and IgG in paired plasma samples or detection of viral RNA by RT-PCR, as described in chapter 2 (section 2.3). All patients or patient's guardian gave written informed consent. Plasma was separated, aliquot and stored at -20^{0}C. One aliquot was sent to the "reference lab" (Dr Suttee Yoksan, Centre for Vaccine Development, Bangkok, Thailand) to determine IgM, IgG levels and classify primary and secondary DENV infection.

3.2.3. The reference assay at the Centre for Vaccine Development, Bangkok, Thailand

The reference anti- DENV IgM and IgG antibody capture ELISAs have been described elsewhere and were performed by a staff of the Centre for Vaccine Development, Bangkok, Thailand [114]. For paired specimens, an increase from less than 15 units of IgM against dengue virus to more than 30 units was considered evidence of an acute dengue infection. A dengue IgM: IgG ratio at discharge equal to or greater than 1.7 defined as primary dengue infection, otherwise defined as secondary infection.

3.2.4. The in-house assay at the Hospital for Tropical Diseases

The in house anti-dengue virus IgM and IgG tests were performed by a single analyst following the procedure described in chapter 2 (section 2.4.1). Results of the samples were reported in units that reflect the fraction of IgM or IgG reactive with the test antigen relative to negative control plasma samples. Results were also reported as negative or positive. Samples with equivocal results were considered as negative if the retest results were still equivocal.

3.2.5. Statistical methods for devising the classification model

Logistic regression was used to model the probability of having primary acute dengue infection. The model was designed based on six covariates: IgM, IgG ELISA units measured in paired plasma samples (enrolment and discharge samples) together with the day of illness at the time these samples were collected. A stepwise backward variable selection procedure was employed to find independent predictors of primary infection with p-to-remove > 0.1. Once the final model was constructed, the rounded coefficients from the model were used to define a diagnostic score (DS). An individual was classified as having primary infection if the probability of primary infection was estimated to be > 0.5.

The accuracy of the proposed model was evaluated by resubstitution (apparent and bootstrap method) and by using an external test data set from a further 93 patients

enrolled in the same manner. The sensitivity, specificity, negative and positive predictive values were calculated and compared. The evaluations were based on patients who were classified as primary or secondary acute dengue infections according to the reference laboratory assay results.

3.3. Results

3.3.1. Evaluation of the HTD in-house anti-DENV IgM ELISA

The evaluation reference panel consisted of 313 well-characterized serum specimens (Table 3-1) collected by a TDR/WHO/PDVI sponsored consortia as described previously [108]. According to the results of reference laboratories, there were 184 samples positive for IgM against DENV and 129 IgM negative samples. The positive samples were obtained from patients with primary (N=29) and secondary (N=155) infections with all four DENV serotypes represented; 28 infected with DENV-1, 19 DENV-2, 14 DENV-3 and 8 DENV-4. The negative samples included samples taken from virologically proven dengue cases with negative IgM serology (N=14), samples from patients with other confirmed infectious diseases (N=84), other diseases (N=4) and from healthy persons (N=27) resident in non-endemic dengue countries.

Table 3-1: Summaries of serum specimens used for evaluation.

Evaluation panel	Number of samples
DENV IgM positive	
primary infections	29
secondary infections	155
Total DENV IgM positive	**184***
DENV IgM negative	
DENV+/IgM-	14 **
Related flavivirus	
West Nile virus	26
Yellow fever virus	10
St. Louis encephalitis virus	2
Japanese encephalitis virus	8
Febrile illness	
Malaria	31
New World hantavirus IgM positive	7
Systemic conditions	
Rheumatoid factor	2
Systemic lupus erythematosus	2
Healthy persons***	27
Total DENV IgM negative	**129**

DENV, dengue virus; Ig, immunoglobulin.

** Includes samples with virological information: 28 DENV-1, 19 DENV-2, 14 DENV-3, and 8 DENV-4. The serotype was not identified for 115 samples.*

*** Includes samples with virological information: 7 DENV-1, 5 DENV-2, 1 DENV-3, and 1 DENV-4.*

**** From areas where dengue is not endemic.*

Diagnostic accuracy of the HTD in house anti- DENV IgM ELISA

Overall, the in-house IgM test correctly diagnosed 94.6% (95% CI 90.3% - 97.4%) of IgM positive specimens and 91.5% (95% CI 85.3% - 95.7%) of IgM negative specimens. The negative and positive predictive values were high; 92.2% (95% CI 86.1% - 96.2%) and 94.1% (95% CI 89.7% - 97%), respectively. Overall, the in-house assay performs comparably with the best available commercial assay tested in the recent TDR/PDVI evaluation [108] (Table 3-2).

Table 3-2: Accuracy of the HTD in-house test with some commercial tests.

Elisa test from	Sensitivity (95% CI)	Specificity (95% CI)
Panbio Diagnostics (Windsor, Queensland, Australia)	99.0% (98.4%–99.5%)	90.6% 88.9%–92.3%
Focus Diagnostics (Cypress, CA, USA)	98.6% (98.0%– 99.2%)	77.8% 75.5%–80.1%,
Standard Diagnostics (Kyonggi-do, South Korea)	97.6% (96.8%–98.4%)	90.0% (88%.3–91.7%)
The HTD in-house test	94.6% (90.3% - 97.4%)	91.5% (85.3% - 95.7%)

Influence of serological responses and dengue virus serotype

The sensitivities of the test layered by primary, secondary and each DENV serotype are shown in table 3-3. The sensitivity was lower in primary dengue infection than in secondary dengue infection but it was not statistical different; sensitivity of 93.1% in primary compared with of 94.8% in secondary DENV infection (P =0.7). Similarly, there wasn't any statistical difference in sensitivity across the 4 serotypes of dengue virus, although the number of DENV-4 samples was small. This suggests that the sensitivity of the in-house anti-DENV IgM ELISA is affected by neither serological responses nor serotype of the dengue virus.

Table 3-3: Sensitivity by serological responses and dengue virus serotype of the in-house test for detection of anti-DENV IgM

	No. positive / total	%	95%CI	*P**
Primary	27/29	93.1	77.2 – 99.2	0.7
Secondary	147/155	94.8	90.1 – 97.7	
DEN-1	27/28	96.4	81.7 – 99.9	0.3
DEN-2	17/19	89.5	66.9 – 98.6	
DEN-3	13/14	92.9	66.1 – 99.8	
DEN-4	6/8	75	34.9 - 96	

** Fisher's exact test.*

False-positive reactions in the HTD in-house IgM assay

The in-house test performance when samples from non dengue- patients or when samples from true dengue patients with undetectable IgM levels were tested is shown in table 3-4. The majority of samples from non-dengue cases demonstrated negative results with the test, except samples from malaria cases, where strikingly 32.3% of samples gave a false-positive result. Amongst samples from true dengue cases but without measurable IgM, only one DENV-1 confirmed sample was positive with the test, representing a 14.3% false-positive rate. This suggests the in-house IgM test was very specific in detecting IgM of DENV infections rather than other flaviviruses infections or other diseases except malaria. Further studies are needed to explain the cause of this cross-reactivity in malaria.

Table 3-4: False- positive rate of the in-house test compared with reference laboratory results for anti-DENV IgM antibody detection.

Evaluation panel	No. of samples	No. of false-positive	False-positive rate (%)
DENV+/IgM-			
DENV-1/ IgM negative	7	1	14.3
DENV-2/ IgM negative	5	0	-
DENV-3/ IgM negative	1	0	-
DENV-4/ IgM negative	1	0	-
Related flavivirus			
West Nile virus	26	0	-
Yellow fever virus	10	0	-
St. Louis encephalitis virus	2	0	-
Japanese encephalitis virus	8	0	-
Other febrile illness			
Malaria	31	10	32.3
New World hantavirus IgM positive	7	0	-
Systemic conditions			
Rheumatoid factor	2	0	-
Systemic lupus erythematosus	2	0	-
Healthy persons	27	0	-

3.3.2. Design of a classification model to classify primary and secondary DENV infection based on the in-house IgM and IgG ELISA test

We hypothesized that the in-house anti-DENV IgM and IgG tests could be used to discriminate between primary and secondary serological responses. To address this hypothesis, we measured IgM and IgG levels in paired plasma samples from a population of Vietnamese dengue cases in which a reference laboratory had a previously made a determination of primary or secondary dengue using the same plasma samples.

The study population

The panel used to generate a classification model consisted of paired plasma samples from 575 laboratory-confirmed dengue cases. Of these, 509 (88.5%) cases were classified primary and secondary dengue by the reference laboratory; 66 (11.5%) cases couldn't be classified in the reference laboratory and were excluded from this analysis. A summary of the demographic characteristics of these patients is shown in Table 3-5. The male: female ratio was 1.6 and the number of cases of secondary infection was higher than primary infection. There was a significant difference in the distribution of age between primary and secondary infection ($P<0.001$, Pearson Chi-square test), with secondary infection more prevalent in the older age groups, but there was no correlation between gender and serological responses ($P=0.2$, Fisher's Exact test).

Table 3-5: Baseline characteristics of the patients.

Age (years)	Gender		Serological responses[a]		Total
	Male	Female	Primary	Secondary	
4-9	49 (50.5%)	48 (49.5%)	33 (34%)	64 (66%)	97 (19.1%)
10-12	79 (60.3%)	52 (39.7%)	34 (26%)	97 (74%)	131 (25.7%)
13-15	79 (64.2%)	44 (35.8%)	35 (28.5%)	88 (71.5%)	123 (24.2%)
16-24	75 (69.4%)	33 (30.6%)	13 (12%)	95 (88%)	108 (21.2%)
25-44	31 (62%)	19 (38%)	0	50 (100%)	50 (9.8%)
Total	313 (61.5%)	196 (38.5%)	115 (22.6%)	394 (77.4%)	509

[a] *Primary and secondary infection was designated by the reference laboratory.*
Values expressed as number (percentage within age group)

Plasma samples from four hundred sixteen cases were randomly selected as the training data set to devise a classification model; of these, 102 were primary infections and 314 were secondary infections based on the results from the reference laboratory. An independent test data set consisted of the remaining cases; of these, 13 had primary infection and 80 had secondary infection, according to the reference laboratory's results. All samples were evaluated for levels of anti- DENV IgM and

IgG antibodies by using the in-house IgM and IgG tests. The infecting DENV serotype was determined by RT-PCR and the serotypes present in the two sample sets is shown in Table 3-6. In total, DENV-1 was the most prevalent serotype, accounting for 51.1% of all dengue confirmed cases. The second most common was DENV-2 (23.5%), then DENV-3 (17.3%). DENV-4 infection was detected in only 6 patients (1.2%). This pattern of serotype prevalence was seen in both data sets.

Table 3-6: Infecting DENV serotype in the study population.

Group	Negative	DENV-1	DENV-2	DENV-3	DENV-4	Total
Training data set	31 (7.5%)	211 (50.6%)	101 (24.3%)	69 (16.6%)	4 (1%)	416
Test data set	4 (4.3%)	49 (52.7%)	19 (20.4%)	19 (20.4%)	2 (2.2%)	93
Total (%)	35 (6.9%)	260 (51.1%)	120 (23.5%)	88 (17.3%)	6 (1.2%)	509

IgM and IgG levels of patients in the training data set

Figure 3-1 shows the levels of anti-DENV IgM and IgG antibodies in relation to the day of illness when the samples were collected, with stratification according the reference laboratory classification of primary or secondary infection. Generally, in primary dengue, IgM levels in the in-house assay were higher than IgG levels, whilst in secondary dengue the reverse was true. During the first 4 days of the illness, DENV-specific IgM and IgG antibodies were detected at low levels in either primary or secondary infection. IgM and IgG antibody levels increased rapidly between days 5-11. Significant higher IgM levels were observed in primary cases on day 6 ($P< 0.01$), 7, 8, 9 (all $P< 0.0001$), and 10 ($P=0.04$) of the illness compared to secondary cases. On the other hand, IgG levels were significantly higher in secondary than primary infection, with the differences at day 6, 7, 8, 9 and 10 significant at $P< 0.0001$.

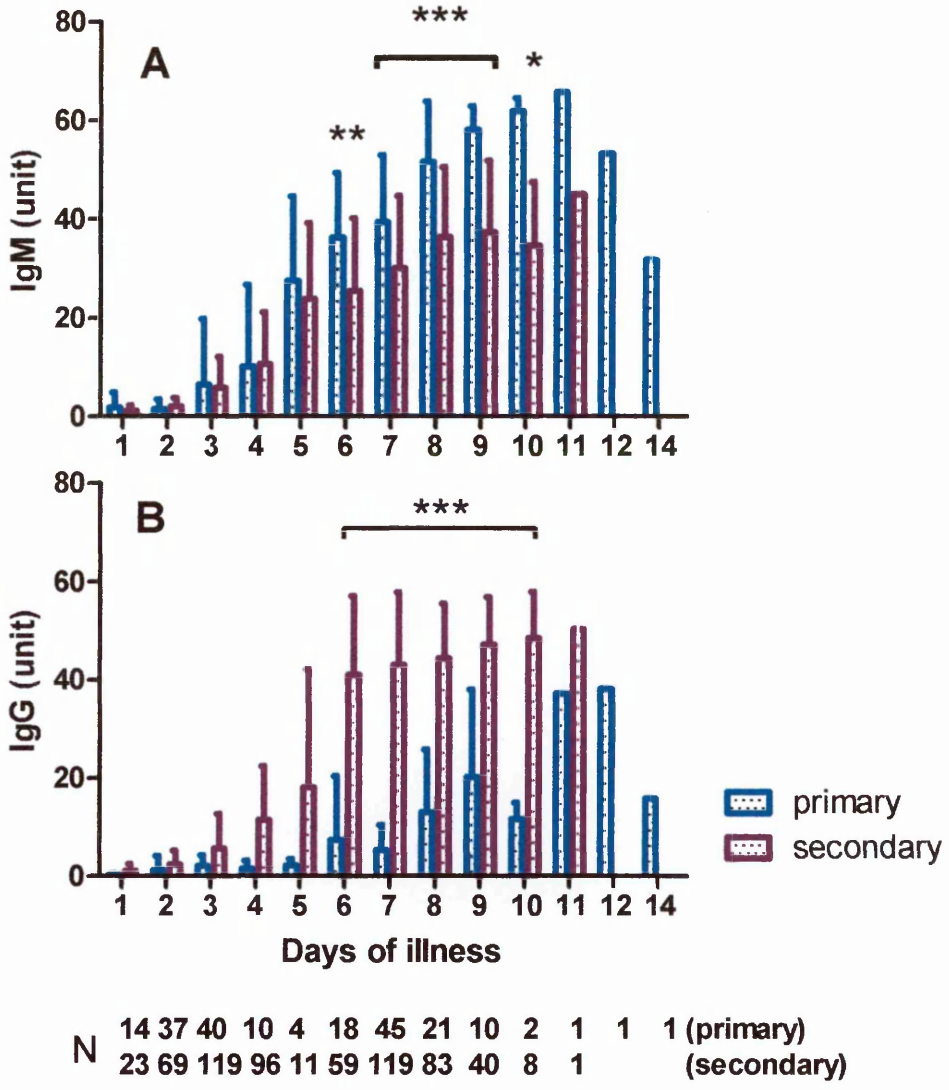


Figure 3-1: Dynamics of anti-DENV IgM and IgG antibody levels in acute and early convalescent-phase plasma samples from dengue patients with primary or secondary dengue by day of illness.

Mean and standard deviation of anti-DENV IgM and IgG levels are shown in (A) and (B), respectively. Blue bars represent primary dengue and red bars are secondary dengue. There were significant differences between primary and secondary at day 6, 7, 8, 9 and 10 days of the illness, P<0.05 (*), <0.001 (**) and <0.0001 (***) (ANOVA, Bonferroni posttest).

To further explore the relationship within the IgM and IgG levels measured by the in-house assays on the acute and early convalescent samples, we performed correlation analysis. Generally, anti-DENV IgM antibody levels were significantly correlated with anti-DENV IgG antibody levels ($P<0.001$, Spearman), though the correlations were weak ($r =0.6$). If using early convalescent samples only, there were two distinct populations of primary and secondary infections with the correlation rates of $r=0.5$ and $r=0.1$, respectively ($p<0.01$, Spearman) but this phenomenon was not seen if using enrolment samples (Figure 3-2).

Collectively, the results suggested that IgM and IgG levels of the early convalescent samples measured by the in-house ELISAs have potentially discriminative features to classify primary and secondary dengue virus infection.

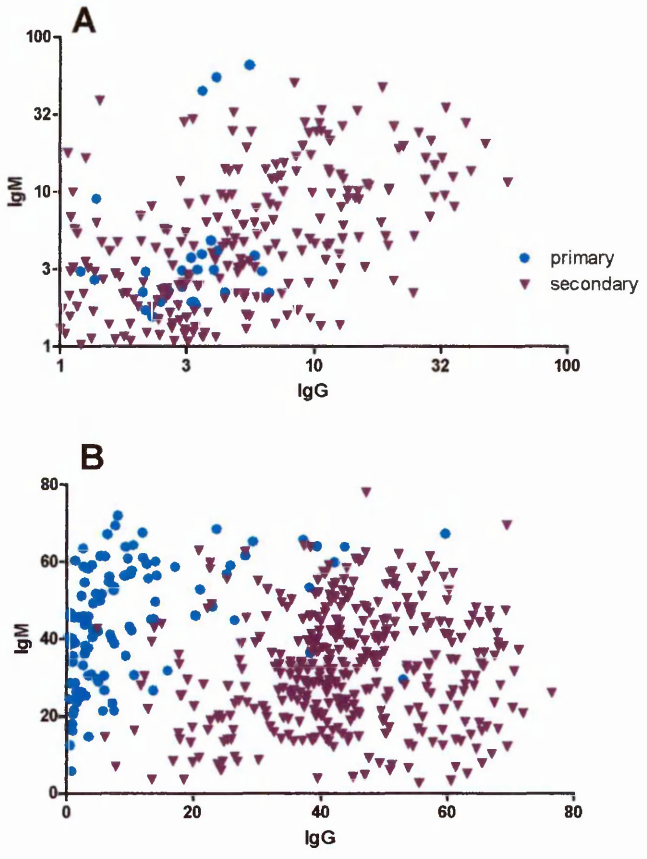


Figure 3-2: Correlation plots of DENV-reactive IgG and IgM antibody levels measured in acute and early convalescent samples by serological responses.

Each point represents the IgM and IgG measurement attained with either enrolment (A) or early convalescent samples (B) using the in-house ELISAs. Blue points represent primary DENV infections, and red points are secondary DENV infections according to the reference assay. IgM or IgG shown in (A) are logarit scales, those in (B) are linear scales. The best distinction between primary and secondary infections was observed when IgM and IgG levels of early convalescent samples were considered compared with those of acute samples.

Generation of a classification model

Because of the difference between the enrolment and the early convalescent samples, we considered them as different variables (IgM of enrolment/ convalescent, IgG of enrolment/ convalescent and day of illness at enrolment/ convalescent). All these variables of the training data set were put into the stepwise logistic regression model to find independent predictors of primary infection with p-to-remove > 0.1. The analysis found 3 variables which were independently associated with a diagnosis of primary infection. The final model is represented in table 3-7.

Table 3-7: The final logistic regression model for the diagnosis of primary infection.

Primary	Coefficient	95% CI	*P*
$IgM_{convalescent}$	0.09	0.05 – 0.13	<0.0001
$IgG_{convalescent}$	-0.21	-0.26 - -0.16	<0.0001
$DOI_{convalescent}$	0.61	0.13 – 1.1	0.01
Constant	-4	-7.08 - -0.9	0.01

DOI: day of illness when the samples were collected.

The classification model for diagnosis of primary infection was derived from the final logistic regression model by rounding the coefficients. An individual was classified as having primary infection if the probability of having primary infection was estimated to be > 0.5. Of note, this cut-off implies that we implicitly qualified the cost of a false positive classification as same as the cost of a false negative classification. So the classification score (CS) was calculated for each patient according to the formula:

$$CS = 6x\ DOI_{convalescent} - 2x\ IgG_{convalescent} + IgM_{convalescent}$$

The cut-off of the classification model was a value of 40. If an individual had a CS of greater than 40, the individual was diagnosed as primary dengue, otherwise as secondary dengue. Using the proposed model gave an excellent discrimination between primary and secondary dengue (AUROC = 0.98, Figure 3-3).

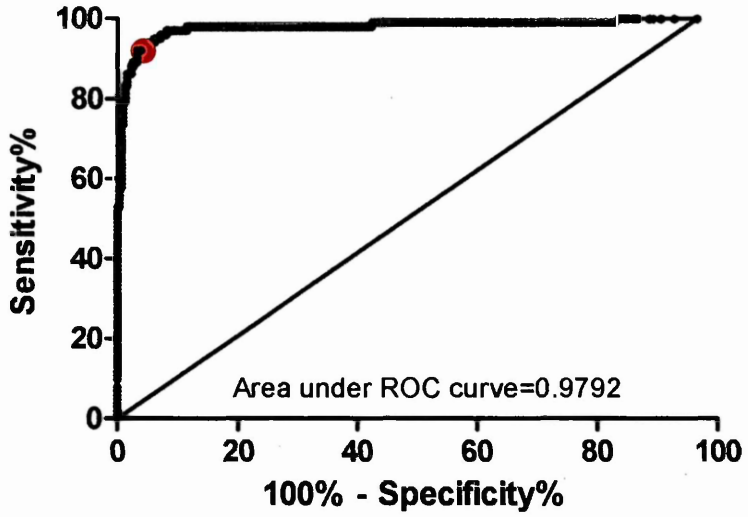


Figure 3-3: Receiver Operating Characteristic (ROC) curve of the model.

ROC curve generated from the logistic regression model for discrimination of primary from secondary acute DENV infection using IgM, IgG levels and DOI measured at early convalescence phase. The red point is the chosen cut-off level.

Validation of the proposed model using the training data set

The performance of the proposed model was estimated using the training data set itself to derive apparent and optimism-corrected estimates (summarized in Table 3-8). Resubstitution misclassified 19 cases (8 primary and 11 secondary cases) giving 92.2% sensitivity, 96.5% specificity, 89.5% positive predictive value and 97.4% negative predictive value. The validation by bootstrap method also gave similar sensitivity, specificity, negative and positive predictive values. Overall, the test correctly diagnosed 95.1% of cases.

Table 3-8: Performance of the proposed model for the diagnosis of primary acute dengue infection.

Accuracy of the test	Apparent*	Optimism-corrected **
Sensitivity	92.2% (94/102)	92.1%
Specificity	96.5% (303/314)	96.2%
Positive predictive value	89.5% (94/105)	88.8%
Negative predictive value	97.4% (303/311)	97.3%
Correctly classified	95.4% (397/416)	95.1%
Area under ROC curve	0.98	0.98

*Accuracy of the test was calculated by * resubstitution or ** bootstrap method using the training data set that generated the model.*

Validation of the proposed model using the test data set.

The accuracy of the proposed model in the diagnosis of primary infection was also tested on an independent set of samples, obtained from 93 dengue patients (in the test data set). Of these, 13 cases were classified as primary infection and 80 cases were secondary infection according to the reference laboratory results. The infecting serotypes in these cases are shown in Table 3-6. The accuracy of the classification model is shown in Table 3-9. The model misclassified only 4 out of 93 cases (1 primary case, 3 secondary cases) and was 92.3% sensitive and 96.3% specific.

Table 3-9: Overall sensitivity and specificity of the proposed model using the test data set.

Accuracy of the test	%	95% CI
Sensitivity	92.3 (12/13)	63.9 – 99.8
Specificity	96.3 (77/80)	89.4 – 99.2
Negative predictive value	98.7 (77/78)	93.1 – 1
Positive predictive value	80 (12/15)	51.9 – 95.7
Correctly classify	95.7 (89/93)	89.4 – 98.8
Area under ROC curve	0.97	0.93 – 1

We also used the test data set to break-down the estimates of accuracy depending on different ranges of illness day at discharge, serological status or the infecting DENV serotypes (Table 3-10). The estimates varied with categories, however there were no significant difference between them ($P>0.5$, Chi-square test), except with serology status ($P=0.02$). This showed that the accuracy of the model in detection of primary cases was not influenced by day of illness when the early convalescent samples were collected nor DENV serotype. However, the specificity of the model would be higher in a group of patients who had detectable IgM and IgG.

Table 3-10: Break-down of the accuracy of the proposed model.

Categories	Sensitivity		Specificity	
	% (N)	95%CI	% (N)	95%CI
Days of illness				
4-7	100 (5/5)	47.8 – 100	96.2 (25/26)	80.4 – 99.9
8-11	87.5 (7/8)	47.3 – 99.7	96.3 (52/54)	87.3 – 99.5
Serology status^{+}				
IgM+/ IgG-	100 (10/10)	69.2 – 100	66.7 (2/3)	9.4 – 99.2
IgM+/ IgG+	-	-	96.3 (77/80)	89.4 – 99.2
DENV serotype				
DENV -ve	-	-	75 (3/4)	19.4 – 99.4
DENV-1	90.9 (10/11)	58.7 – 99.8	94.7 (36/38)	82.3 – 99.4
DENV-2	-	-	100 (19/19)	82.4 – 100*
DENV-3	100 (2/2)	15.8 – 100*	100 (17/17)	80.5 – 100*
DENV-4	-	-	100 (2/2)	15.8 – 100*

N: number test observed / total number. CI: confidence interval. $^{+}$ *P=0.02, comparing specificity between IgM+/IgG- and IgM+/IgG+.* one- sided, 97.5% CI.*

3.4. Discussion

Accurate dengue diagnosis is important for case management, surveillance, outbreak investigation and research. Whilst the laboratory confirmation of dengue cases can sometimes be difficult depending on i/ the number of the days which the patient has had the symptoms and ii/ which diagnostic tests that are accurate, economic, easy to handle and speed are available. Results of this chapter showed the accuracy of the HTD in-house anti-DENV IgM ELISA in detecting low levels of IgM in plasma samples, and the HTD in-house anti-DENV IgM and IgG ELISAs could be used for distinction between primary and secondary acute dengue. Accordingly, these methods and classifier model are being used for the routine serodiagnosis of dengue in patients enrolled in research studies conducted by the OUCRU and HTD.

Commercially manufacturered anti-DENV IgM ELISA tests are widely available but only a few of them have adequate sensitivity and specificity for use in

dengue diagnosis especially for cases with low IgM response [108, 203]. Indeed, one of the common problems with these assays is the occurance of false-positive results on sera from patients with malaria, rheumatoid factor, Japanese encephalitis, or leptospirosis [107, 108, 203]. The HTD in-house IgM assay showed 94.6% sensitive and 91.5% specificity in detecting anti-DENV IgM when compared with the reference laboratory results and it did not have any cross-reactivity with other flaviviruses. Moreover, the sensitivity of the test was equal across four DENV serotypes as well as primary and secondary infections.

The in-house test failed to detect 10 samples that were determined by the reference test as being IgM positive and 11 samples that were IgM negative. Reasons may be because of differences between the two methods. Firstly, antigens were different between them. The in-house method used culture supernatants harvested from C6/36 cells infected with each of four dengue serotypes instead of sucrose acetone-extracted suckling mouse brains injected with prototype vaccine. Secondly, difference in the calculation of results. The reference laboratory reported results in units that reflected the fraction of IgM and IgG reactive with the DENV antigen relative to positive standards while the units of the in-house method were the fraction relative to negative standards.

A further limitation of the in-house IgM test was false-positive results with sera from malaria cases. This is consistent with findings from other MAC ELISA assays, which are also prone to giving false-positive results from malaria cases [108]. The explanation may be that they share some epitopes. Since DENV and Plasmodium parasites co-circulate in some parts of Southeast Asia, including in some parts of central Viet Nam. It's important to understand this limitation of the MAC ELISA in this regard. An alternative explanation is that false positive reactions occurred because malaria elicits polyclonal B cell plasmacytosis resulting in the generation of many low avidity IgM antibodies. Some of which may bind non-specifically with DENV antigens.

We also observed a small false-positive rate (14.3%) in a serum sample that was DENV IgM negative according to the reference test but came from an acute dengue patient with DENV-1 infection. The simplest explanation here is that the in-house assay was more sensitive than the reference assay on this sample.

Collectively, these results suggest the in-house IgM assay is a robust method for the detection of anti-DENV IgM and therefore allows the presumptive laboratory diagnosis of dengue, albeit usually after 3-4 days of fever. Due attention is required to the fact that false-positive IgM results are possible in malaria cases and therefore it would be good patient management to include a malaria blood smear for patients whose history or clinical presentation suggests that malaria is a possible diagnosis.

Many methods have been proposed to differentiate primary from secondary DENV infections. The HI assay, based on antibody tittering of paired or single serum specimens has been recommended by WHO for this purpose [1]. The plaque reduction neutralizing test may be also used but is known to be an assay that requires stringent standardization [204]. The challenges around the use of these two assays in resource limited settings are to find i/ a suitable cell line and viruses which can give visible results (suspension or plaque) and ii/ right endpoint titers that can meet the criteria for virus activity (a percentage reduction of virus activity). The major drawbacks of these techniques are difficulty to perform, time to conduct and cross-reactions among flaviviruses [109, 199, 205].

Since the development of antibody-capture ELISAs, an IgM/IgG ratio of above 1.7 or IgM elevated early while IgG appeared later with low titer has been used to define a primary case using a single serum specimen [114-118]. The difficulty with using a simple ratio of 1.7 is that it is highly unlikely to be translatable to antibody-capture ELISAs that are performed in different laboratories under different environmental conditions, with different analysts and with different reagents and plasticware. Moreover, this cut-off approach does not take into account the day of illness on which the samples were collected, and because the IgM/IgG response is dynamic, the day of illness must be considered as an important factor.

To our knowledge only two approaches have factored the day of illness in the classification of primary versus secondary dengue. Matheus et al described an IgG avidity test using a single acute-phase serum sample for the discrimination of primary from secondary dengue and included the day of illness as a variable [112, 113]. Not long ago, a 2-D classifier model based on anti-DENV IgG units for the classification of primary and secondary DENV infections was reported [111]. Their results showed sensitivities of 82.7% and 91.6% respectively and specificities of 77.5% and 92.5%,

respectively. However, their sample size used to generate the models was small (50 and 109 patients, respectively) and serum samples were mainly from adult patients [111]. Besides, the cross-reactivity of flavivirus- specific IgG antibodies wasn't considered in these methods.

Collectively, our classification model, a 3-D classifier, took advantage of IgM and IgG capture ELISA data together with the illness day of the sample collection to distinguish primary from secondary dengue. The test method described assays a single dilution of plasma and results were reported in units. This method appears to be superior to other assays in which titers are measured using serial dilution of samples. There is no requirement for specimen pretreatment and only a small volume of sample (10μl) is required.

To generate the classification model, the results from serology assays in the reference laboratory at the Centre for Vaccine Development (Bangkok) were used. We selected this laboratory to generate the reference results because it is widely recognized internationally as a centre of excellence in dengue diagnosis and indeed is the main Asian dengue reference laboratory in the WHO/TDR dengue diagnostic network. The reference laboratory couldn't determine the serological status of 66 cases, and these cases were excluded from our analysis. These virologically-confirmed dengue cases had undetectable anti-DENV IgM and IgG levels in the reference lab. One explanation for this is that the antibody-capture ELISA used in the reference laboratory is less sensitive than our in-house assay. This situation might lead to the classification model weighted toward populations that had higher levels of IgM and/or IgG.

In conclusion, our data showed plasma collected within the first week of the illness could be used for dengue diagnosis as well as the differentiation between primary and secondary DENV infections. The classification model worked well with discharge samples on day 4 onward of the illness and the in-house tests gave greater than 90% sensitivity and specificity if we compared with the reference laboratory results. These sensitivity and specificity values were consistent across serology status, dengue serotype and day of illness. On the back of these results, the HTD in-house ELISAs and the 3-D classifier has now become the routine serological method of dengue diagnosis in the HTD.

4.NS1 in dengue - a diagnostic tool which may help in prognosis

4.1. Introduction

DSS typically occurs between day 4 and 6 of illness. There are no reliable mechanisms in the first few days of illness of identifying patients who will develop DSS. Several studies have explored the risk factors associated with the progression of disease from mild disease, dengue fever (DF), to severe disease, dengue haemorrhagic fever (DHF) and/or dengue shock syndrome (DSS) [9, 97, 206-208]. Issues related to age, immune status [208, 209], population genetics [210], viraemia titers, distinct serotypes [97, 126], early NS1 concentrations [39, 41] and the proinflammatory cytokines elicited [169, 186, 188] were investigated and had different degrees of correlation with severe dengue.

Commercially available NS1 detection tests have been widely used as dengue diagnostic tools to complement the existing approaches of PCR, serology and, less frequently, virus isolation [211-214]. In the first 3 days of illness, sensitivity and specificity of the Platelia NS1 tests were ~80% and 100% respectively in laboratory-confirmed Vietnamese paediatric dengue cases [99, 103, 105, 215]. Soluble NS1 levels were higher in serum in the first 72hrs of fever in Thai children who developed DHF, leading to the suggesting that NS1 concentrations could be prognostic for severe dengue [39, 41]. Nevertheless the potential for early NS1 plasma concentrations to predict severe clinical outcomes (e.g. DHF/DSS) has not been systematically assessed.

The aims of this chapter are to examine whether plasma levels of NS1 in the first 4 days of fever can be used as a prognostic marker of DSS. This chapter also investigates the kinetics of NS1 and viraemia in children with DSS and milder presentations of dengue.

4.2. Methods

4.2.1. Study design and patient recruitment

There were two patient populations for two purposes. The first population provided data for a "training" data set in which the purpose was to identify a threshold of NS1 concentration that could predict DSS progression. The second patient population represented the "testing" set which was used to independently evaluate the results generated in the training data set.

The training patient population was paediatric inpatients at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. These children were enrolled into a prospective clinical study of dengue if there was a clinical suspicion of dengue and the children had a fever history of 4 days or less. The children were enrolled in the hospital ward. Plasma samples from these patients were collected at the time of enrolment and the time of discharge from hospital.

A second patient population was enrolled at Dong Thap Hospital, Dong Thap province, Viet Nam. The inclusion criteria included patients who were both NS1 positive and had the illness duration of no more than 4 days. Patients underwent screening in the outpatient clinic if there was a clinical suspicion of dengue and the illness duration of no more than 4 days. Enrolment plasma samples from eligible patients were tested with the Platelia dengue NS1 Ag test (Biorad, California, USA). All patients that were NS1 ELISA positive were admitted for further observation and participation in this prospective, hospital-based, study of dengue in Dong Thap hospital. Plasma samples from these patients were collected at the time of study enrolment, then every two days until they were discharged from hospital. A subset of these patients who had DENV-1 or DENV-2 infection identified by RT-PCR was selected to provide the test data set. The disease severity classification was based on the WHO classification [72] (section 2.2 in Chapter 2). The study protocols were approved by the Oxford Tropical Research Ethics Committee and Scientific and Ethical Committee of Hospital for Tropical Diseases and Dong Thap Hospital. The parents/guardians of all participants gave written informed consent for their children to participate in the study.

4.2.2. Serological responses

IgM and IgG against DENV were measured by capture ELISA in plasma samples collected at enrolment and at the time of hospital discharge using the method described in section 2.4.1 in Chapter 2). Primary infections or secondary infections were determined by using the classification model described in Chapter 3.

4.2.3. NS1 quantitative ELISAs

To measure concentrations of plasma NS1 Ag, the DENV NS1 Ag Platelia ELISA kit was used as previously described (section 2.4.2 in Chapter 2). The NS1 values were expressed as ng/ml if using the absolute-quantitative method or shown in NS1 Ag Bio-Rad units (BRU/ml) if using the semi-quantitative method.

Absolute-quantitative ELISA

The quantitative assay procedure was performed following the manufacturer's instructions. To calculate the concentration of plasma NS1 Ag, two-fold serial dilutions of recombinant DENV-2 NS1 Ag (supplied by Hawaii Biotech) were run in duplicate on each plate to establish standard curves in the assay together with the serially diluted plasma samples (from neat to 1000 dilution in 10-fold dilution). The standard curve was diluted from 1ng/ml to 0.03ng/ml. Samples with levels above the maximum optical density were diluted more and had to be retested. The NS1 concentrations of samples were expressed as nanogram per milliliter (ng/ml). The limit of this detection was 0.05ng/ml.

Semi-quantitative ELISA

The plasma NS1 levels were expressed in NS1 Ag Bio-Rad Units per milliliter (BRU/ml) by using the same reagents and assay procedure that were used in the original ELISA (NS1 qualitative ELISA, section 2.4.2 in Chapter 2). If samples had OD<3, the NS1 Ag BRU/ml of the samples corresponded to the sample ratio [Sample NS1 (BRU/ml) =sample OD/cut-off OD]. If samples had OD>3, the assay procedure was repeated with a 1:5000 dilution of the conjugate instead of 1:50 dilution. The NS1 Ag BRU/ml of the samples were calculated by multiplying the OD of the re-tested samples by 150 [Sample NS1 (BRU/ml) =sample OD*150/cut-off OD].

4.2.4. Statistical methods

Comparisons of plasma NS1 concentrations between serotypes (6 comparisons) or between serological statuses within serotypes (4 comparisons) were based on linear regression models adjusted with day of illness when the samples were collected. We corrected for multiple comparisons using a single step approach based on the joint asymptotic multivariate distribution of the contrasts [216].

4.3. Results

4.3.1. Patient population

The baseline characteristics of the study patients are shown in Table 4-1. There were 566 suspected pediatric dengue patients enrolled into the study at HTD from May 2006 to October 2007. Among these, according to the dengue laboratory algorithm, 497 patients had laboratory confirmed dengue, 50 of them were excluded from the analysis because their day of illness were more than four days from the illness onset, 6 had recent DENV infections, 9 cases had recent flavivirus infections and the remaining (54 cases) were "other febrile illnesses". Among laboratory-confirmed dengue cases, 22 patients developed DSS at some time after their enrolment and were transferred to the intensive care unit. The remaining 425 patients who didn't progress to DSS were managed throughout in the general dengue ward (described here as uncomplicated dengue patients – UC dengue). There were no differences in age, sex, serological status or day of illness between UC dengue and DSS patients on study entry.

Compared with laboratory diagnosis of dengue cases by either RT-PCR or serology test, the NS1 Platelia test had a sensitivity of 80.1% (95%CI, 76% – 83.7%) and specificity of 96.7% (95%CI, 91.4% – 99%). Among the 447 laboratory confirmed dengue cases (either by RT-PCR, NS1 or serology test), the DENV RT-PCR results were positive for 415 (92.8%) cases. Secondary infection was diagnosed in 343 (76.7%) cases. NS1 was undetectable in all patients with OFIs, while the proportion of UC dengue and DSS cases to be NS1 positive was 80% (340/425) and 86.4% (19/22), respectively ($p>0.05$).

Table 4-1: Baseline table summarizing key clinical, viral and antigenic information of the study population.

Variable	UC dengue (N =425)	DSS (N =22)	*P*
Male sex	268 (63.1%)	16 (72.7%)	0.3
age(years)	11 (5 – 15)	11 (6 – 14)	0.4
Day of illness at enrolment	3 (1 – 4)	3 (1 – 4)	0.07
2	59 (13.9%)	2 (9.1%)	
3	177 (41.6%)	12 (54.5%)	
4	188 (44.2%)	7 (31.8%)	
Serological status*			0.3
Primary infection	98 (23.1%)	3 (13.6%)	
Secondary infection	325 (76.5%)	19 (86.4%)	
Infecting serotype			0.2
DENV-1	234 (51.1%)	13 (59.1%)	
DENV-2	113 (26.6%)	9 (40.9%)	
DENV-3	43 (10.1%)	0	
DENV-4	3 (0.7%)	0	
Log10 Viraemia ** (copies/ml)	7.2 (5.9 – 8.2)	7.7 (6.1 – 9)	0.22^{+}
NS1 (+)	340 (80%)	19 (86.4%)	0.6
NS1 (ng/ml)			
All patients	22.7 (0.95 – 292.9)	22.8 (4.6 – 774.3)	<0.0001^{+}
DENV-1	124.5 (5.7 – 436.4)	472 (4.3 – 1276)	0.03
DENV-2	1.3 (0.005 – 7)	7.2 (5.3 – 17.8)	0.02

*Data are expressed as N (%) or median (IQR). UC dengue: uncomplicated dengue, DSS: dengue shock syndrome. * 2 indeterminated results in UC dengue group. ** viraemia was detected in 393 patients in UC dengue.$^{+}$ P adjusted for serotype, serological status and day of illness.*

4.3.2. NS1 concentrations and correlations with viraemia

We quantified plasma NS1 concentrations in patients with a detectable NS1 antigenaemia at enrolment. NS1 concentrations were determined by the semi-quantitative method (reported in BRU/ml) and the absolute-quantitative method (reported in ng/ml) using recombinant NS1 (DENV-2) as a standard. Figure 4-1 describes the correlations between NS1 values and those with viraemia observed in the study population.

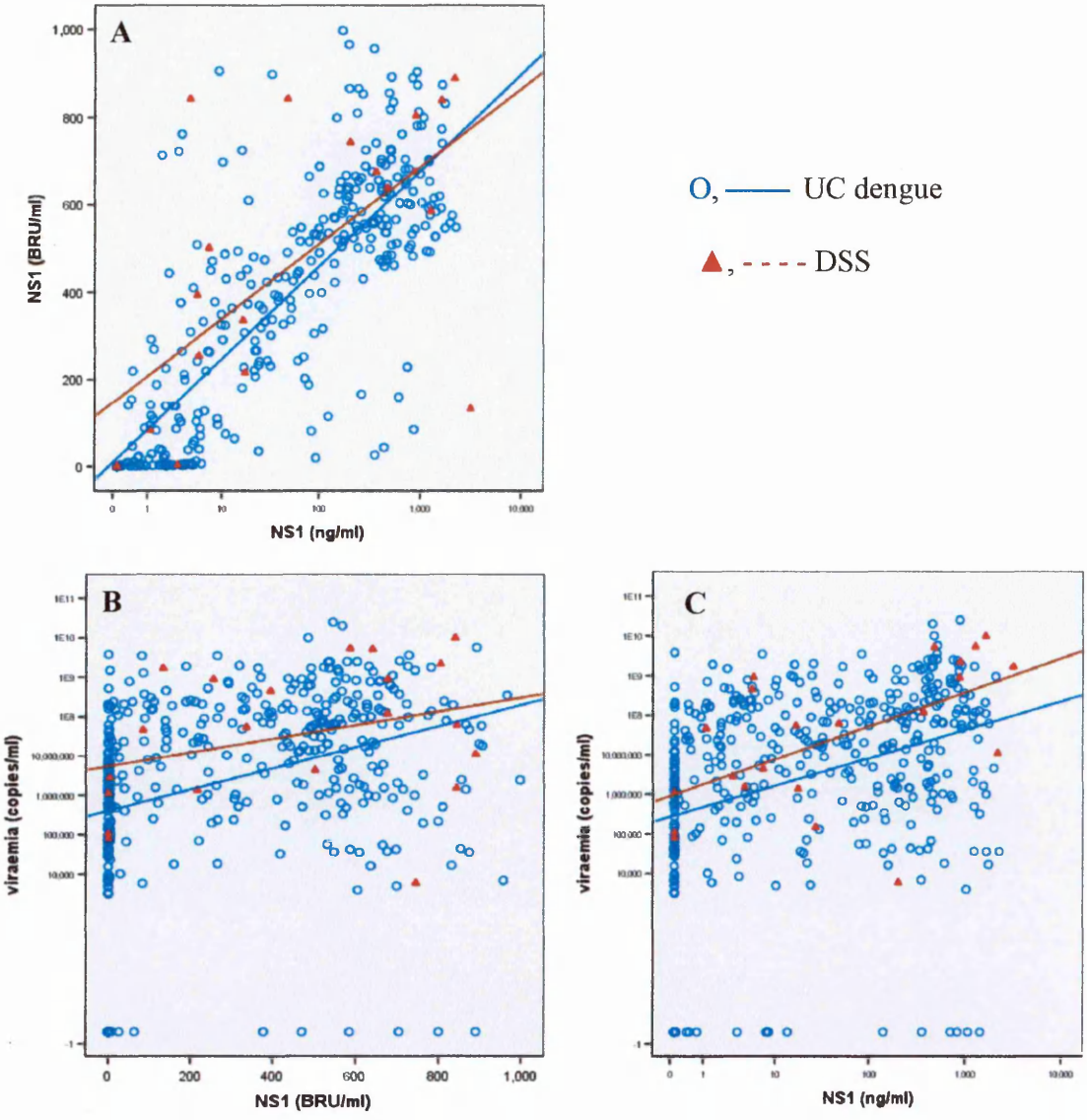


Figure 4-1: Correlations between viraemia and NS1 concentration in UC dengue or DSS patients.

(A) Spearman correlation between log10 NS1 ng/ml and NS1 BRU/ml (r=0.78), (B) correlation between NS1 BRU/ml and log10 viral load (copies/ml) (r=0.3) and (C) correlation between log10 NS1 ng/ml and log10 viral load (copies/ml) (r=0.4) with all p<0.0001 derived from partial correlation after controlling for serotype, serological status and day of illness. DSS patients were shown in red triangle (▲) and their fit line in red; non-DSS patients were shown by blue circle (O) and their fit line in blue.

Overall, the absolute NS1 concentrations highly correlated with values from the semi-quantitative method (partial correlation controlled for serotype, serological status and day of illness with r=0.78, p<0.0001).

Patients who were NS1 negative had significantly lower median viraemia levels than NS1 positive patients with the same serotype, serological status and duration of illness history [log10 viraemia-median (IQR), 5.7 (4.6 – 6.7) vs. 7.6 (6.2 – 8.4), respectively, adjusted p<0.0001] (Figure 4-2). Actually, the NS1 concentrations correlated with viraemia levels in the same group of samples (r=0.4 if NS1 concentrations were reported in ng/ml and r=0.31 if NS1 concentrations were reported in BRU/ml, both p<0.0001, partial correlation controlled for serotype, serological status and day of illness). Besides, similar significant associations were also observed within subgroups of patients with DENV-1 or DENV-2 infection (Table 4-2).

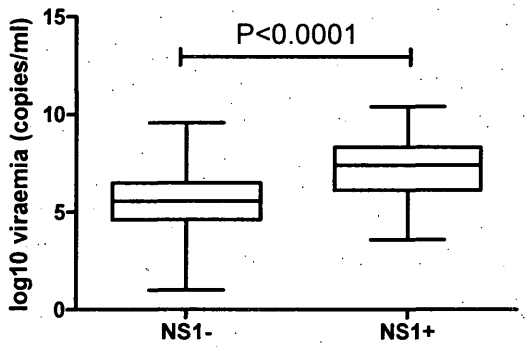


Figure 4-2: Plasma viraemia in patients with NS1 positive or negative

Box and whisker plots of plasma viraemia levels in enrolment samples represent median, 25th and 75th percentile, minimum and maximum of the data classifying by NS1 quanlitative results.

Table 4-2: Partial correlations between plasma NS1 levels and viraemia at enrolment.

Parameter	All serotypes*		DENV-1		DENV-2	
	NS1 (BRU/ml)	Viraemia	NS1 (BRU/ml)	Viraemia	NS1 (BRU/ml)	Viraemia
NS1 log10- (ng/ml)	0.78	0.4	0.76	0.47	0.73	0.48
NS1 (BRU/ml)	-	0.31	-	0.37	-	0.3

Viraemia levels in Log10- (copies/ml) scale. Numbers represent the correlation rate (r), all partial correlations had p<0.0001. P values were derived from partial correlation controlled for serological status, day of illness and (*) serotype was extra added for controlling the correlation.

4.3.3. Differences in plasma NS1 levels between serotypes and serological status

NS1 concentrations at enrolment in the patients stratified by serotype and serological status are presented in Table 4-3. Comparisons between serotypes were done by using logistic regression adjusted for day of illness. This revealed that NS1 levels were significantly higher in DENV-3, followed by DENV-1 and lowest in DENV-2 infections (all adjusted $p<0.05$, in the footnote of Table 4-3). No significant differences were observed in comparisons between DENV-4 and other serotypes due to the small number of DENV-4 infections (n=3). Interestingly, the differences between serotypes disappeared after adjustment for log10-viraemia on the same day (all adjusted $p>0.2$). However, comparisons between serotypes after adjustment for day of illness and for multiple comparisons showed no significant differences in viraemia levels between serotypes (medians of log10-viraemia in DENV-1 to 4 infections were 7.2, 7.4, 6.6 and 5.1, respectively. All adjusted p values>0.2). Comparisons between primary and secondary infections using logistic regression adjusted for day of illness showed non significant differences between primary and secondary serological state among subgroups, although in univariate analysis significantly higher NS1 concentrations were observed in children with primary DENV-1 infection compared with those in children with secondary DENV-1 infection (Figure 4-3).

Table 4-3: Plasma NS1 concentrations in children at enrolment stratified by serotype and serological status.

	DENV-1	DENV-2	DENV-3	DENV-4
All*				
N	247	122	43	3
Median	142.3	1.8	346.3	30.1
IQR	(5.4 – 447.9)	(0 – 8.1)	(21.7 – 1231.5)	(15.4 – 48.1)
Primary				
N	80	6	12	-
Median	191.7	9.9	347	
IQR	(64.5 – 454.9)	(3.6 – 16.1)	(264 – 910.4)	
Secondary				
N	166	115	31	3
Median	80	1.5	319.1	30.1
IQR	(2.6 – 443.6)	(0 – 7.1)	(10.6 – 1384)	(15.4 – 48.1)

** Serological status was indeterminate in 2 patients (1 DENV-1 and 1 DENV-2). Comparisons between serotypes or between serological status were controlled for day of illness, p values were adjusted for multiple comparisons: DENV-1 – DENV-2, DENV-1 – DENV-3, DENV-2 – DENV-3, all p<0.001. DENV-3 – DENV-4, p=0.045. All others p>0.1. If the comparisons between serotypes were adjusted for log10-viraemia on the same day, all p values were >0.25.*

Because DENV-1 or DENV-2 infections represented 88.2% of the serotypes detected, and none of the DENV-3 or DENV-4 infections resulted in DSS, we selected DENV-1 and DENV-2 infected patients for all further analysis. Figure 4-3 shows NS1 concentrations in enrolment plasma of children with DENV-1 or DENV-2 infection stratified by serological status with p values derived from univariate analysis. Among patients with primary or secondary infections, NS1 concentrations (measured by semi- or absolute quantitative methods) were significantly higher in DENV-1 infected patients than DENV-2 infected patients ($p<0.001$, Mann-Whitney test). Besides, among DENV-1 infected patients, plasma NS1 concentrations from primary infections were significantly higher than those from secondary infections. This difference between primary and secondary infections wasn't observed among DENV-2 infections.

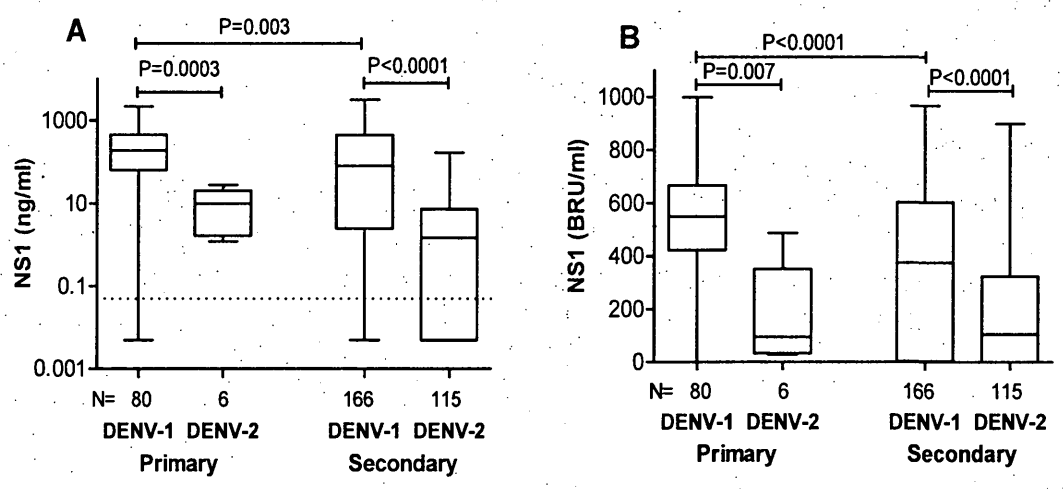


Figure 4-3: Plasma NS1 concentration at enrolment in children with DENV-1 or DENV-2 infection by serological status.

Box and whisker plots of plasma NS1 levels in enrolment samples represent median, 25th and 75th percentile, minimum and maximum of the data classifying by infecting DENV serotype and, serological status. Plasma NS1 concentrations, measured by absolute quantitative method (A) or semi-quantitative method (B), were significantly lower in DENV-2 patients than DENV-1 patients (within primary or secondary infections), and were significantly higher in primary than secondary infections with DENV-1. P values were from Mann-Whitney tests.

4.3.4. Plasma concentrations of NS1 in patients who developed DSS

To understand the interaction between infecting serotype, serological status, day of illness, NS1 concentration and clinical outcome, we performed a multivariate logistic regression to identify variables that were independently associated with DSS (Table 4-4). The result shows that plasma NS1 concentrations in enrolment plasma were significantly lower in UC dengue versus DSS patients [median and inter-quartile range (IQR), 22.7 (0.95-292.9) ng/ml vs. 22.8 (4.6-774.3) ng/ml, respectively, P<0.0001, Table 4-4]. In addition, DENV-1 serotype was also independently associated with DSS (p=0.046).

Table 4-4: Logistic regression model identifying variables associated with DSS

Variable	OR	95% CI	*P*
Day of illness	0.7	0.4 – 1.3	0.26
Primary	0.46	0.1 – 1.9	0.28
DENV-1	0.32	0.1 – 0.98	0.046
NS1 concentration (ng/ml)	1.002	1.001 – 1.003	<0.0001

OR: odd ratio. 95% CI: 95% confident interval.

When we stratified patients by their infecting serotypes, plasma NS1 concentrations at enrolment were significantly higher in DENV-1 infected patients who progressed to DSS than in DENV-1 infected patients who didn't develop DSS [median (IQR), 472 (4.3-1276) vs. 124.5 (5.7-436.4), respectively, p=0.03, Mann-Whitney test] (Figure 4-4). Similarly, NS1 concentrations were significantly higher in DENV-2 infected patients who progressed to DSS than in DENV-2 infected patients with an uncomplicated disease evolution [median (IQR), 7.2 (5.3-17.8) vs. 1.3 (0-7), respectively, p=0.02, Mann-Whitney test] (Figure 4-4).

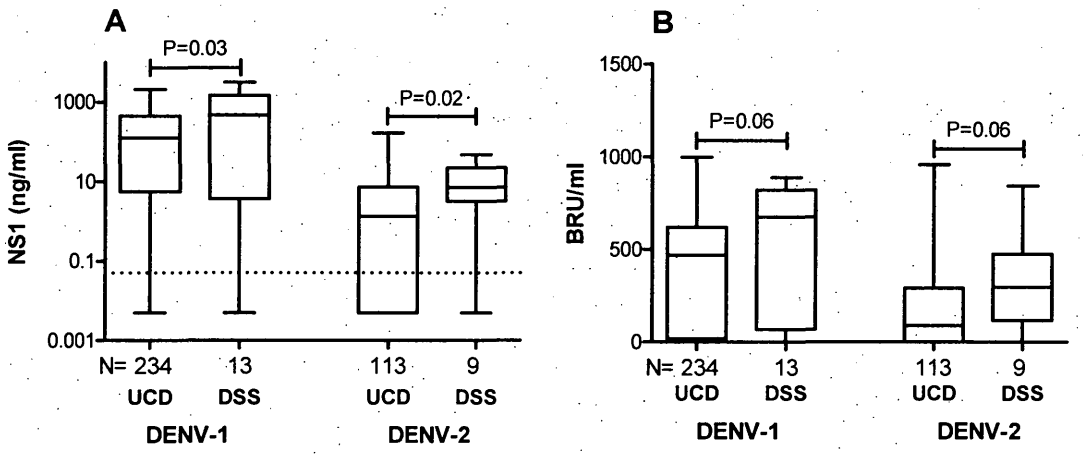


Figure 4-4: Plasma NS1 concentrations at enrolment by disease severity and serotype.

Box and whisker plots of plasma NS1 levels at enrolment represent median, 25th and 75th percentile, minimum and maximum of the data classifying by disease severity. Showing in the graphs are concentrations of plasma NS1 measured by absolute quantitative method (A) or by semi-quantitative method (B). Plasma NS1 levels measured by absolute quantitative method were significantly higher in DSS patient with DENV-1 infections or DENV-2 infections than in UC dengue patients with DENV-1 infections or DENV-2 infections (p= 0.03 or 0.02, respectively, Mann-Whitney test) (A). Significant trends between two groups were also observed when results reported using the semi-quantitative method (p=0.06, Mann-Whitney test). UCD: uncomplicated dengue. DSS: Dengue shock syndrome.

4.3.5. NS1 concentrations in prognosis of DSS

To identify a threshold of plasma NS1 concentration that might be predictive of DSS in the first few days of illness, we analyzed receiver operating characteristic (ROC) curves of NS1 concentrations which were stratified by infecting serotype with DSS as the endpoint (Figure 4-5). Maximum of sensitivity plus specificity was chosen as a cut-off value for each serotype, DENV-1 or DENV-2. Among patients with DENV-1 infection, plasma NS1 concentrations of greater than 875ng/ml on day 1-4 of illness predicted the subsequent development of DSS with a sensitivity of 50% and a specificity of 92.94% (Figure 4-5A). Among DENV-2 infected patients, plasma

NS1 concentrations greater than 6.3ng/ml at enrolment predicted DSS progression with a sensitivity and specificity of 77.8% and 73.4%, respectively (Figure 4-5B).

Using the semi-quantitative NS1 method, plasma NS1 concentrations of greater than 638 BRU/ml on day 1-4 of illness in patients with DENV-1 infection predicted DSS with a sensitivity of 61.5% and a specificity of 78.3% (Figure 4-5C). Among DENV-2 infected patients, plasma NS1 concentrations of greater than 214 BRU/ml at enrolment predicted DSS with 75% sensitivity and 66.95% specificity (Figure 4-5D).

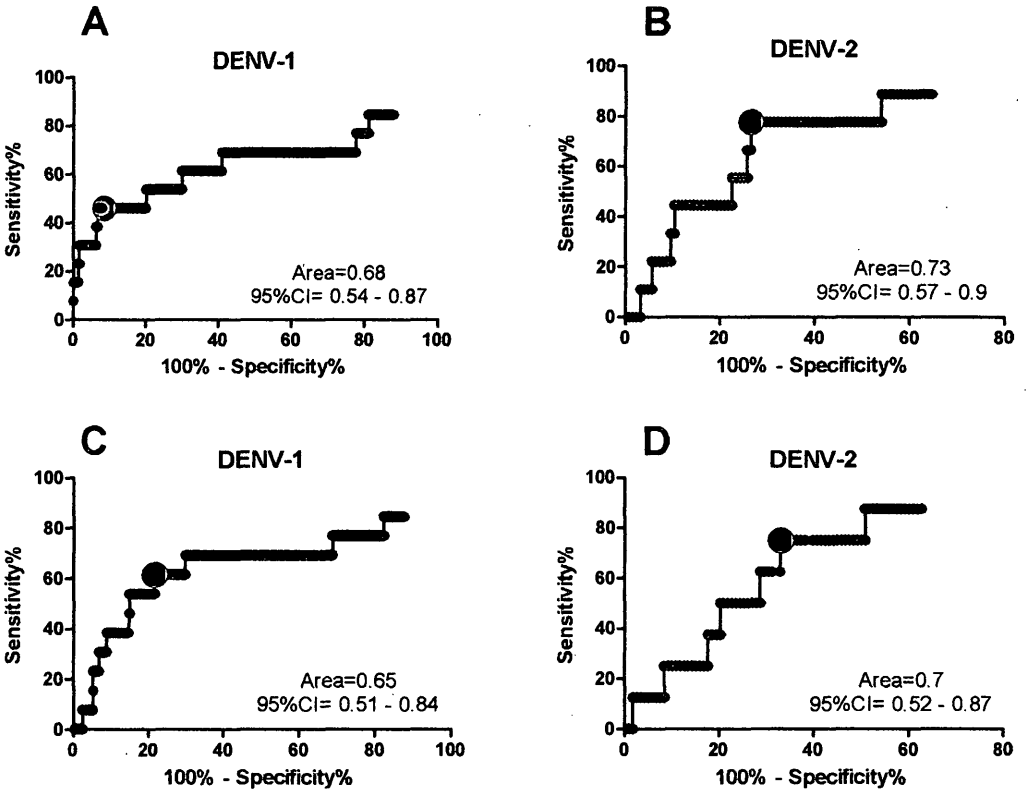


Figure 4-5: ROCs of plasma NS1 concentrations as prognostic marker for DSS progression among DENV-1 and DENV-2 infected patients.

There were 13 patients with DENV-1 who developed DSS and 9 patients with DENV-2 who developed DSS. Data shown in A and B are generated from absolute quantitative method and those in C and D are from semi-quantitative method. Red points represent the chosen cut-off values. 95%CI: 95% central interval range. ROCs: receiver operating characteristic curves.

4.3.6. Evaluation of NS1 cut-off levels for prognosis of DSS

To evaluate the NS1 cut-off values for prognosis of DSS in a second, independent sample set, we used enrolment plasma samples obtained from children recruited into another prospective study in Dong Thap hospital. Between May 2007 and December 2007, we enrolled 316 inpatients with laboratory confirmed dengue (NS1 positive). There were 294 patients who were eligible to enter the test data set (Table 4-5). The patients who were excluded had insufficient volume of plasma samples for quantitative NS1 (n=22). Sex, age, DENV serotypes and day of illness at enrolment were distributed similarly between two patient groups. However, almost all DSS cases had serological evidence of secondary dengue (94.6%) whilst amongst the UC dengue cases, secondary infections accounted for 79.4% of the total (p=0.02, Fisher's Exact test).

Consistent with the first cohort, enrolment plasma NS1 concentration was higher in the DSS group than in the UC dengue group ($p<0.0001$, adjusted for serotype, serological status and day of illness). In univariate analysis, among patients with DENV-1 infections, plasma NS1 concentrations were also significantly higher in the DSS group at the time of enrolment than in UC dengue cases. Amongst DENV-2 infected patients, there was a non-significant trend for higher NS1 concentrations in DSS cases (p=0.08, Table 4-5).

Table 4-5: Baseline table summarizing key clinical, viral and antigenic information of the patients in the test data set

Variable	UCD (N =257)	DSS (N =37)*	*P*
Male sex	138 (53.7%)	25 (67.6%)	0.2
age(years)	11 (7 – 16)	10 (7 – 14)	0.5
Day of illness	3 (2 – 3)	3 (2 – 3)	0.9
2	57 (21.1%)	9 (29.1%)	
3	171 (68.4%)	23 (58.3%)	
4	20 (6.6%)	4 (8.3%)	
Defervescence day	-3 (-4 – -2)	-3 (-3 – -2)	0.7
Serological status			0.02
Primary infection	53 (20.6%)	2 (5.4%)	
Secondary infection	204 (79.4%)	35 (94.6%)	
Infecting serotype			0.5
DENV-1	207 (80.5%)	30 (81.1%)	
DENV-2	35 (13.6%)	6 (16.2%)	
DENV-3	11 (4.3%)	0	
DENV-4	2 (0.8%)	0	
NS1 (+)	257 (100%)	37 (100%)	-
NS1 (ng/ml)			
All patients	145.5 (17.9 – 428)	1002.3 (73 – 1638.3)	<0.0001^{+}
DENV-1	217.4 (52 – 464.5)	1166.4 (666 – 1815.34)	<0.0001
DENV-2	2.4 (1.2 – 7.7)	19.6 (6.1 – 34.9)	0.08

Data are expressed as N (%) or median (IQR). UCD: uncomplicated dengue, DSS: dengue shock syndrome. One DSS patient who had undetectable DENV serotype progressed to shock a day after enrolment. + P adjusted for serotype, serological status and day of illness.*

In this second cohort, the subgroup of 278 patients with DENV-1 (n=237) or DENV-2 (n=41) infection were further analysed. The patients that were excluded from the analysis were those with DENV-3 (n=11) or DENV-4 (n=2) infections or undetectable DENV serotype (n=3). Thirty six of the DENV-1 or DENV-2 patients subsequently progressed to DSS as defined by the WHO criteria and they were treated

for DSS. The remaining 242 patients with DENV-1 or -2 infections were defined as UC dengue cases and they did not require any clinical interventions and were managed in the general infectious disease ward.

We applied the previously determined plasma NS1 cut-off values of 875 ng/ml or 6.3 ng/ml for each serotype, DENV-1 or DENV-2 respectively, to determine its accuracy for the prognosis of DSS. Out of 30 DSS patients with DENV-1 infections, 19 had plasma NS1 concentrations at enrolment that were above the 875 ng/ml threshold, yielding a sensitivity of 63.3% (95%CI, 43.8% – 79.5%). Amongst UC dengue with DENV-1 infection, 25/207 had plasma NS1 concentrations above the 875 ng/ml threshold, giving a specificity of 87.9% (95%CI, 82.5% – 91.9%). The numbers with DENV-2 were small, but nonetheless four out of six DENV-2 infected patients who developed DSS had plasma NS1 concentrations that were above the 6.3 ng/ml threshold The sensitivity and specificity for DENV-2 was 66.7% (95%CI, 24.1% – 94%) and 68.6% (95%CI, 50.6% – 82.6%), respectively (Table 4-6).

Table 4-6: Sensitivity and specificity of the test using cut-off values to predict DSS.

Serotype	Cut-off	No. of positive test	Sensitivity [% (95%CI)]	Specificity [% (95%CI)]
DENV-1	875 (ng/ml)	44	63.3 (43.9 – 79.5)	87.9 (82.5 – 91.9)
	638 (BRU/ml)	66	60 (40.8 – 76.8)	76.8 (70.3 – 82.3)
DENV-2	6.3 (ng/ml)	15	66.7 (24.1 – 94)	68.6 (50.6 – 82.6)
	214 (BRU/ml)	14	66.7 (24.1 – 94)	71.4 (53.5 – 84.8)

When using the semi-quantitative NS1 method, the plasma NS1 cut-off value of 638 BRU/ml was applied to predict DSS progression of DENV-1 infected patients and 214 BRU/ml was used among DENV-2 infected patients. Among patients with DENV-1 infections, there 18 cases tested positive out of 30 DSS patients, the sensitivity was 60% (95%CI, 40.8% – 76.8%) and the specificity was 76.8% (95%CI, 70.3% – 82.3%). Among patients with DENV-2 infections, there were four cases tested positive out of six DSS patients and 10/35 UC dengue patients tested positive,

the sensitivity and specificity were 66.7% (95%CI, 24.1% – 94%) and 71.4% (95%CI, 53.5% – 84.8%), respectively (Table 4-6).

Collectively, these results suggest that NS1 concentrations have potential as prognostic markers of DSS, although surprisingly this has a serotype dependent nature.

4.4. Discussion

A timely diagnosis and an accurate prognosis of severe dengue in the first few days of illness could be helpful for clinical management, disease surveillance and clinical research activities. In this study, we demonstrated that at initial presentation, within 1– 4 days of illness, the plasma NS1 concentrations correlated with viraemia levels and that a NS1 threshold could be used for the prognosis of DSS. This is the first time that NS1 concentrations have been used for the prognosis of DSS.

It's important to point out that the Platelia NS1 Ag test has been evaluated widely [103, 104, 217, 218]. The test has been shown to be suitable for early diagnosis of dengue with high sensitivity (82-93%) and specificity (100%) [103, 104, 217], and could be considered as first-line testing for acute dengue diagnosis in a single serum sample [104, 217]. However, the test has some limitations: the sensitivity of the test was reportedly lower in DENV-2-infected patients than DENV-1 or DENV-3 infected patients [103, 105, 219] and the sensitivity was also lower in those with secondary infections relative to primary infections [103-105, 217-219].

In our study, we had three NS1 negative patients who developed DSS and those patients all had secondary infections (2 DENV-1 and 1 DENV-2 infection, viraemia $<10^6$ copies/ml). The inabiliy to detect NS1 might be due to the lower sensitivity of the test with secondary infection and perhaps reflects the presence of NS1 specific-antibody in secondary immune responses [212]. This could lead to the clearance of NS1 or else NS1 would bind to the antibodies to form immune complexes so that the target epitopes were not recognized by mAbs in the NS1 ELISA test. Another possible reason for undetectable NS1 in those DSS cases might be that most of the soluble NS1 had bound to endothelial cells in tissues, such as in the lung and liver [129]. A final possibility is that the self-reported illness history for these patients was inaccurate and this is suggested by the low viraemia detected in these

cases ($<10^6$ copies/ml).

We demonstrated that at initial presentation, the plasma NS1 concentration correlated with viraemia levels. This observation is consistent with previous findings in Vietnamese infants [219] and children and adults [103, 105, 220] and also in other patient groups [39, 41, 127]. However, the strength of the correlation between NS1 and viraemia as measured by qRT-PCR was not particularly strong in our study and in this regard it was similar to the study by Duyen *et. al* [220] (partial correlation r=0.4 and 0.6, respectively). One reason for this was the difference in methods. Unlike NS1 detection by NS1 ELISA, the detection of RNA by RT-PCR was not influenced by the presence of DENV-IgG antibodies in the test samples. The direct detection of viraemia by RT-PCR shares a similar window to that of NS1. However it has become apparent that there was not a precise correlation between the two markers, with NS1 often detected in the absence of RNA and vice versa [98, 221, 222]. Although originally viewed as a surrogate marker for viraemia, circulating levels of NS1 are perhaps the measure of infected cell mass. Degradation and/or clearance from circulation of these two products from infected cells are also likely to vary [219].

Duyen *et. al.* reported that NS1 levels among patients with DENV-1 infections were higher in primary than secondary infections; and DENV-1 infections were associated with higher plasma NS1 concentrations than DENV-2 infections even after adjusting for viraemia [220]. Consistent with this, we observed that plasma NS1 concentrations were higher in DENV-1 infected patients than those in patients with DENV-2 infections, but the difference was absent after adjusting for viraemia on the same day. Possible reasons to explain the difference in NS1 levels between DENV-1 and DENV-2 are differences in viraemia burden and/or different avidity of binding to NS1 from DENV-1 and DENV-2 by Mabs in the commercial Platelia assay kit.

Plasma NS1 concentrations at enrolment were significantly higher in children who subsequently developed DSS than in UC dengue patients. This is consistent with the observation that higher levels of NS1 were found in the plasma of a small number of Thai children with DHF/DSS during the early febrile phase than in children with DF [39, 41]. Avirutnan *et. al.* proposed that complement activation mediated by NS1 may contribute to pathogenesis of the vascular leakage of DHF/DSS patients [41] although later he has also observed *in vitro* that NS1 could protect DENV from

complement-dependent neutralization in solution due to the binding of NS1 to C4 and inhibition of complement activation [128]. NS1 binding to C4 could directly limit opsonization and neutralization of virions, thereby facilitating local dissemination.

We identified cut-off concentrations of NS1 that could detect DSS patients with DENV-1 or DENV-2 infections. The specificities of our cut-off values were lower in the test data population because the training data set included NS1 positive and NS1 negative patients, while the test data set recruited only NS1 positive cases. Thus, if the test data set contained NS1 positive and NS1 negative patients the specificities of the proposed NS1 levels for predicting patients who progress to DSS could be much higher.

A previous study showed that a free NS1 concentration of above 600ng/ml on presentation predicted the subsequent development of DHF with a sensitivity of 72% and a specificity of 79% [39]. These numbers were derived from a small number of subjects with secondary DENV-2 infections (n=32). Our cut-off, sensitivity and specificity for DENV-2 serotype were lower than those of Libraty *et. al.*'s study possibly because they used a DENV-2 specific MAbs and an in-house assay for detecting DENV-2 NS1 antigen, while our method was based on a commercially available NS1 test.

Our study had several limitations. First limitation is that this study was biased to patients admitted to hospital whereas the majority of DENV infections resulted in clinically inapparent or mild disease that are managed entirely in the community. Second, we only focused on DENV-1 and DENV-2 because DSS wasn't seen among DENV-3 and DENV-4 infections and the number of patients who were infected with DENV-3 and DENV-4 was small. Third, the quantitative NS1 method used a standard from DENV-2 which might have less efficiency of being recognized by Mabs in the commercial Platelia assay kit. And the final limitation is that the second study population contained only NS1 positive patients.

There were significant limitations in using the cut-off NS1 concentrations for DSS prognosis. First, not all patients who developed DSS had detectable NS1 in their acute phase plasma, either because of viraemia declining or immune-complexes forming. Indeed, it was recognized that the sensitivity of NS1 detection was reduced in patients with secondary infection [103-105, 217-219]. And serotyping of DENV

infection was an expensive and time-consuming method that is rarely performed as a routine diagnostic in endemic settings and therefore the serotype dependent nature of the cut off values is a serious hurdle to its practical application.

In brief, the research showed that there was a sturdy correlation between the absolute and semi-quantitative methods for calculation of plasma NS1 concentration and NS1 concentration in enrolment plasma sample of patients could be useful for predicting DSS in patient with DENV-1 or DENV-2 infection but it was only useful alongside information on the infecting DENV serotype.

5. Early clinical and laboratory features predictive of dengue haemorrhagic fever

5.1. Introduction

The initial symptoms of dengue –commonly, fever, headache, myalgia, and malaise– are nonspecific and similar to those of other viral illnesses. This can create challenges in both clinical management and disease surveillance. DHF is uncommon given the total number of infections in endemic areas, but it is a severe presentation of DENV infection and requires hospitalization and adequate management. Thus, early clinical diagnosis of dengue and especially the detection of early clinical signs and symptoms of DHF are difficult but necessary for improving patient triage and management. The WHO guidelines for diagnosing DHF rely on laboratory and clinical signs that are usually not present in the first few days of illness. The definitive diagnosis of DHF is made only with the development of thrombocytopenia and haemoconcentration (or pleural effusion), which usually occurs around the time of defervescence [194].

Attempts to identify clinical features as well as early laboratory features for prognosis of DHF have been made in many hospitals and laboratories [69, 124, 169, 182-184, 223-225]. Such studies provided a list of markers that showed correlations with disease severity at early illness, but their application in early prognosis of DHF were not apparent because they didn't establish cut-offs for predict DHF.

Several hypotheses have been proposed to explain the pathogenesis of severe DENV infection. Some have suggested a role for high viraemia levels and delay in the clearance of viraemia in the pathogenesis of DHF [97, 124, 125]. Others have nominated a critical role for cytokines in DHF pathogenesis [164-172]. The most likely scenario is that DHF pathogenesis is multifactorial and that virus and host immune responses are contributing factors.

The principal aims of this study were to define the early clinical presentation and laboratory features of DHF in a large prospective cohort of patients under 18 years old admitted to Dong Thap (DT) and Tien Giang (TG) hospitals in Vietnam within 72 hours of their illness, and to identify some clinical signs, symptoms or laboratory biomarkers that can be used to predict the severity of dengue or may assist in differentiating DHF from DF or from other common febrile illnesses.

5.2. Methods

5.2.1. Study setting

Recruitment took place at Dong Thap (DT) (Dong Thap Province) and Tien Giang (TG) (Tien Giang province) hospitals (both located in southern Vietnam). Patients eligible for enrolment were between 18 months and 18 years of age, with a fever history of less than 72 hours and the treating doctor believed dengue was a possible diagnosis. All patients or their guardians provided written informed consent. Ethical approval was from the Scientific and Ethical Committee of DT and TG hospitals and the Oxford Tropical Research Ethical Committee. Patients were seen daily by the hospital's physicians until they were clinically well enough to be discharged from hospital. Detailed clinical and hematological information were recorded every day in a standard case report form.

Each patient had a venous blood sample drawn on the day of enrolment and daily thereafter during hospitalization until discharge. Plasma was separated, aliquoted and stored at -20^{0}C. Serological assays (IgM/IgG ELISA), RT-PCR and NS1 ELISA (Platelia, BioRad) were used to confirm all dengue cases.

After completion of the case records, a single physician, who was not directly involved in patient care, assigned a final diagnosis of DF, DHF or OFI based upon the WHO guidelines [72].

5.2.2. Serological status, viraemia and cytokine measurements

IgM and IgG against DENV were measured in all patients' plasma at enrolment and discharge by anti-DENV IgM/ IgG ELISAs (described in section 2.4.1 of chapter 2). Primary and secondary infections of the patients were determined by using the model described in chapter 3.

DENV RNA was detected by RT-PCR (described in section 2.4.4 of chapter 2) in enrolment plasma samples. For those patients who were DENV RT-PCR positive, viraemia were measured in all of their serial daily plasma samples by the

qRT-PCR assay (described in section 2.4.5 of chapter 2). Every individual sample contained an internal control and external controls were used in every assay.

Patients who had positive results with all PCR, NS1 and serology tests were chosen to measure cytokine concentrations. Cytokine measurement was performed on their enrolment and discharge plasma samples using the method described in section 2.4.6 of chapter 2.

5.2.3. Data record

Case Report Forms (CRFs, Appendix 7) were filled in by doctors at DT/TG hospital. Data were then entered and managed by the Data entry department of OUCRU.

5.2.4. Statistical methods

We aimed to detect early markers for DHF (DHF vs. DF/OFIs) by examining a pre-defined list of 7 laboratory and 13 clinical covariates. We first performed univariate summaries of covariates in the two groups and compared them using the Wilcoxon rank-sum test for continuous and Fisher's exact test for categorical data. We then aimed to develop predictive models for DHF based on all clinical or/and laboratory covariates, respectively. We used logistic regression for model development and report the full model and a model with stepwise model selection according to the Bayesian information criteria (BIC, [226]). The performance of the selected models was then calculated at the cut-off value at which probability of DHF progression is greater than 20%. The validation (including the model selection process) was based on bootstrap method (with a 100 time repeated process) to overcome the optimism from resubstitution.

To compare viraemia and cytokine measurements between disease severity (two groups: DF and DHF), we pre-defined the following contrasts to be of primary importance: pairwise comparisons of total (DF vs. DHF), of DENV serotypes within disease severity (9 comparisons, e.g. DF with DENV-1 vs. DHF with DENV-1, DF with DENV-1 vs. DF with DENV-2, etc.), and comparisons of serological statuses within disease severity (4 comparisons, e.g. DF with primary vs. DHF with primary, DF with secondary vs. DHF with secondary, DF with primary vs. DF with secondary

and DHF with primary vs. DHF with secondary). Comparisons of (log-transformed) viraemia and cytokine measurements were based on linear regression models adjusted with day of illness when the samples collected and with serotype (in comparisons of serological status within disease severity) or serological status (in comparisons of serotypes within disease severity) or both serotype and serological status (in comparisons of total). Comparison of the time to viral clearance was based on a Cox regression model. In all cases, we included dummy covariates for each serotype-disease severity combination (e.g. D1.DF means DF patient with DENV-1 infection, etc.) or serological status-disease severity combination (e.g. P.DF means DF patient with primary infection, etc.) and then tested for significance of the pre-specified contrasts. We corrected for multiple comparisons using a single step approach based on the joint asymptotic multivariate distribution of the contrasts; this method serves the same purpose as the Bonferroni correction but is more efficient [216].

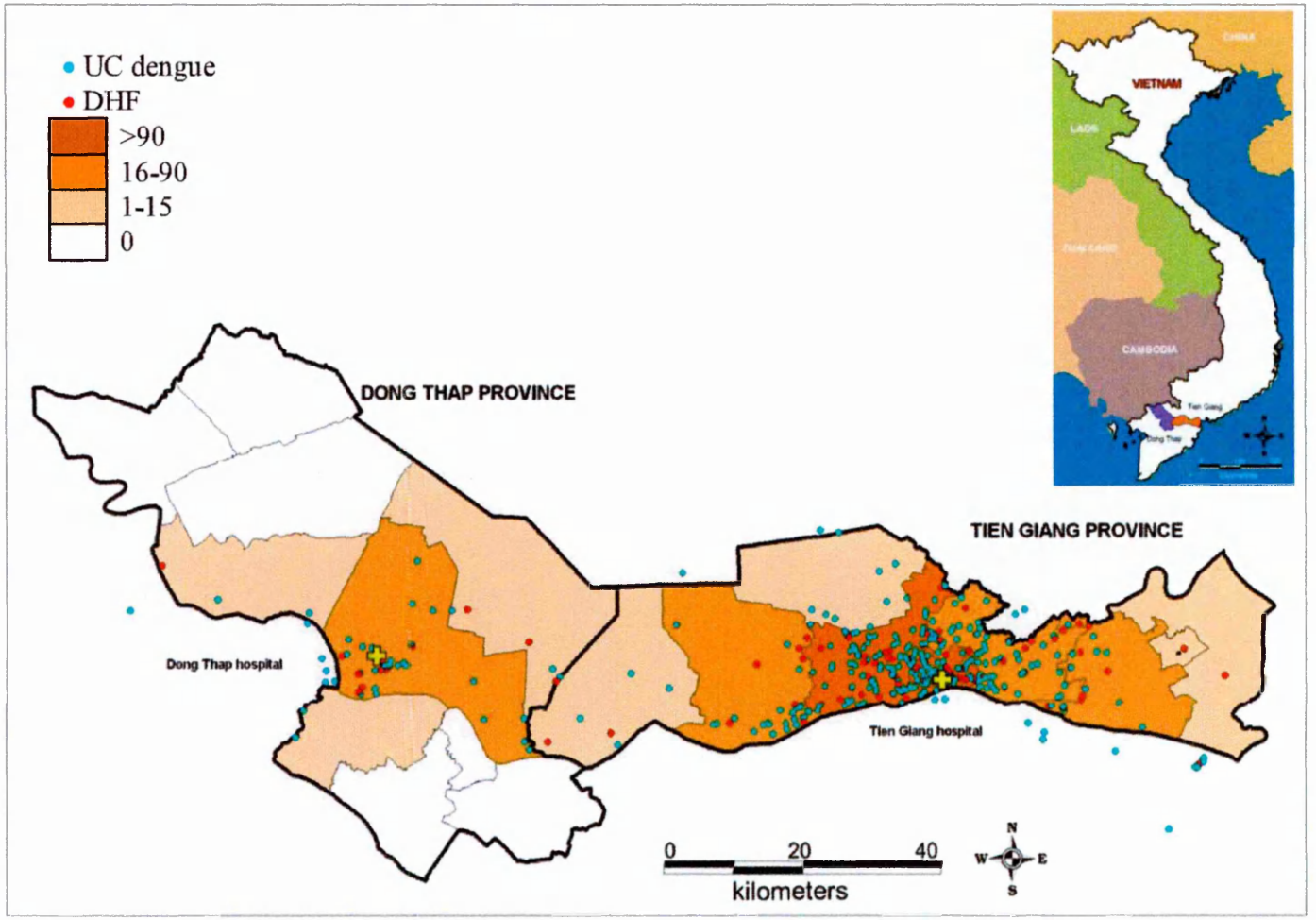
We used the statistical software R version 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria) and the companion R packages BMA 3.13 (Bayesian Model Averaging) and RPART 3.1-46 (Recursive partitioning and regression trees) for analyses. Missing values were imputed by using the companion R packages mice 2.1.

5.3. Results

5.3.1. Number of patients and laboratory diagnosis

Between December 2007 and November 2009, 574 patients were enrolled in the study at Dong Thap hospital (n=61) and Tien Giang hospital (n=513). Seventeen patients were excluded from the analysis, 14 of them had incomplete hospital information records and 3 entered the cohort later than 72 hours from the self-reported illness onset. Of those which were included, according to our dengue laboratory algorithm (Figure 2-1 in section 2.3), 333 (59.8%) patients had laboratory confirmed dengue, 22 (3.9%) had serological evidence for a recent DENV infection (n=16) or for a recent flavivirus infection (n=6). Among the remaining cases, 179 (32.1%) were conclusively negative on all diagnostic tests (defined as other febrile illness – OFI), and 23 (4.1%) had indeterminate results.

Figure 5-1 shows the geographic locations of study patient addresses in DT and TG provinces, which were deduced by GPS logging of each patient's residential address. The majority of patients lived in close proximity to the study hospitals and there was no evidence of any particular spatial clustering of dengue cases, which is reflective of the endemic nature of dengue in southern Vietnam.



The right corner map shows the location of Vietnam in the Southeast Asian region; highlighted in blue and red are DT and TG provinces, respectively. The main map shows DT and TG provinces. Provinces are separated by dark black boundaries and districts are separated by light black boundaries. Locations of DT and TG hospital are marked with yellow crosses. The color scale shows the number of patients enrolled in the study from each district. Each dot shows the residential address of a study patient. Red dots are DHF cases and light blue dots are UC dengue cases.

Figure 5-1: Geographic distribution of study patients' residential addresses at DT and TG province.

Amongst cases with acute DENV infections, 272 (81.7%) patients were RT-PCR positive in the enrolment plasma sample, an extra 43 (12.9%) had NS1 Ag recognized by qualitative NS1 ELISA and a further 18 (5.4%) had IgM/IgG seroconversions by IgM/IgG ELISA (Figure 5-2).

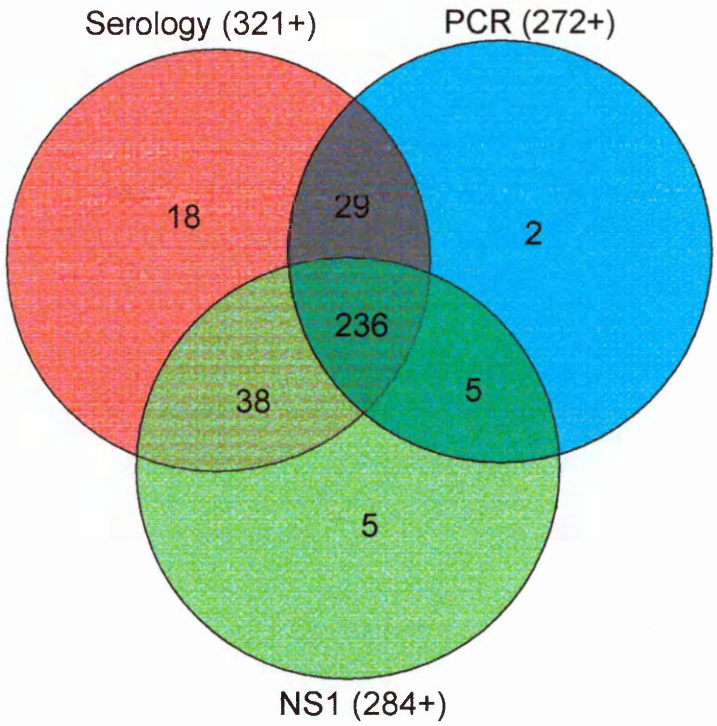


Figure 5-2: Summary of results from the three diagnostic tests.

The venn diagram shows 333 laboratory confirmed acute dengue cases according to whether they tested positive by RT-PCR, NS1, or/and serological tests. Blue represents PCR+ test; green is NS1+ test and pink represents IgM or IgG seroconversions.

5.3.2. Demographics and disease severity in the study population

For the present analysis, we included all OFI cases (n=179) and 318 laboratory confirmed dengue cases; we excluded 15 cases (4.5%) because a final clinical classification of DF or DHF could not be determined by the WHO criteria [72]. Among the laboratory confirmed dengue cases, the characteristics of the study population are shown in table 5-1. The median age of patients enrolled in Dong Thap hospital was higher than those in Tien Giang hospital (10 vs. 9 years old, respectively, p=0.04). There were no significant differences in male to female ratio, duration of fever before study entry or proportion of patients with DHF between the two study sites. There were more females in the DHF group than the group of DF and OFI patients (54.1% vs. 43.3%, p=0.05, Chi-square). Children were older in the DHF group than the group of DF and OFI patients [median (IQR) age of 11 (7-14) vs. 9 (6-12) years, respectively, p=0.001].

Table 5-1: Study population characteristics by final diagnosis.

Diagnosis	OFI	DF	DHF I	DHF II	DHF III	Total
n (% of total)	179 (36)	209 (42.1)	10 (2)	84 (16.9)	15 (3)	497 (100)
Age (years)	7 (5–11)	10 (7.5–13)	6.5 (6–10.5)	12 (7.5–15)	9 (6–13)	9 (6–13)
Male, n (%)	102 (57)	118 (56.5)	4(40)	40 (47.6)	6 (40)	270 (54.3)
DOI	2 (2–3)	3 (2–3)	3 (2–3)	3 (2–3)	3 (2–3)	3 (2–3)
Defervescence day	-2 (-3 – -1)	-3 (-4 – -2)	-3 (-4 – -2)	-3 (-4 – -2)	-3 (-4 – -2)	-4 (-6 – -3)
Length of hospitalize (day)	5 (4–6)	5 (4–6)	5 (4–7)	5 (5–6)	6 (6–7)	5 (4–6)

Values are shown in either number (% of total) or median (IQR, 25th-75th quartile range). DOI: day of illness.

5.3.3. Infecting serotype and serological status

Plasma samples collected from patients at enrolment were analyzed by RT-PCR as described previously (section 2.4.4). This enabled determination of the infecting DENV serotype in viraemic patients. The DENV RT-PCR was positive for 265 of 318 dengue patients (83.3%); 45 of 53 RT-PCR negative patients (84.9%) had secondary infections. DENV-1 (52.8%) was the most prevalent serotype in the study population; the second most common serotype was DENV-3 (18.2%), then DENV-2 (11.6%). DENV-4 infection was detected in only two cases (Table 5-2). The proportion of DENV serotypes was similar between DF and DHF groups (p=0.3, Chi-square).

The majority of dengue patients had secondary infection (n=250, 78.6%), primary infection was more common in DENV-1 infections (Table 5-2). Secondary infections were more common in DHF patients all grades than in DF patients (99/109, 90.8% vs. 151/209, 72.2%, respectively, $p<0.001$, Fisher's Exact). Among the children with DENV-2, 3 or 4 infection, the distribution of secondary infection between groups was similar; but among DENV-1 infected patients, the proportion of patients with secondary infection was significant higher in DHF patients than DF group (52/59, 88.1% in DHF group vs. 68/109, 62.4% in DF group, p=0.001, Fisher's Exact).

Table 5-2: Infecting serotype and serological status by diagnosis.

Diagnosis		Undetectable DENV	DENV-1	DENV-2	DENV-3	DENV-4	**Total**
Serological status	Primary infection	8 (15.1)	48 (28.6)	0	12 (20.7)	0	68 (21.4)
	Secondary infection	45 (84.9)	120 (71.4)	37 (100)	46 (79.3)	2 (100)	250 (78.6)
	Total	53 (16.7)	168 (52.8)	37 (11.6)	58 (18.2)	2 (0.6)	318
Final diagnosis	**DF/OFI**						
	N	211	109	22	44	2	388*
	Secondary	26 (12.3)	68 (62.4)	22 (100)	33 (75)	2 (100)	151 (38.9)
	DHF all grade						
	N	21	59	15	14	0	109
	Secondary	19 (90.5)	52 (88.1)	15 (100)	13 (92.9)	0	99 (90.8)
	Total	232 (46.7)	168 (33.8)	37 (7.4)	58 (11.7)	2 (0.4)	497

Values are shown in number (% of total). number includes DF (n=209) and OFI (n=179)*

5.3.4. Patient management

Each patient stayed a median of 5 (IQR 4-6) days in hospital until they were discharged from hospital. 113/497 (22.7%) of study patients received some intravenous fluid replacement. The frequency of parenteral fluid intervention given to patients who did not have dengue (17/179, 9.5%) was significantly lower than that of laboratory confirmed dengue patients (96/318, 30.2%) at $p<0.0001$ (Chi-square test). Fluid was primarily for shock resuscitation (14 DHF III cases, 1 OFI), for rehydration (9 OFI, 36 DF, 3 DHF I, 35 DHF II, and 1 DHF III) or for maintenance only (6 OFI, 2 DF, 6 DHF II). Three cases were given inotropes (2 DHF III, 1 DHF I), 3 were given diuretics (all DHF III) and 4 needed oxygen support (1 DHF II, 3 DHF III).

5.3.5. Clinical signs and symptoms at enrolment

The most common clinical symptoms and signs in dengue patients at presentation were high body temperature (median of 39^0C, IQR 38^0C – 39^0C), vomiting, bleeding, flush, abdominal pain and tenderness. To identify early clinical markers of DHF, we compared signs or symptoms in DHF patients at the time of presentation with the rest of the patient population (DF and OFI). Vomiting, spontaneous bleeding, tenderness and liver enlargement at presentation were significantly associated with DHF by univariate analysis ($P<0.05$). Gender and age were also associated with DHF (Table 5-3). Spontaneous bleeding, especially skin bleeding which was the most common bleeding sign seen in this study, had a significant correlation with DHF (OR 2.2, 95%CI 1.4-3.4, $P<0.0001$).

Table 5-3: Demographic and clinical signs and symptoms at enrolment that were associated with progression to dengue hemorrhagic fever.

Variable	Total	Dengue hemorrhagic fever				
		N / median	% / IQR	OR	95% CI	P
Total	497	109		-	-	-
Age (median-IQR)	**497**	**11**	**7-14**	**1.09**	**1.04-1.15**	**0.001**
Gender (female)	**227**	**59**	**26**	**1.54**	**0.99-2.42**	**0.05**
BMI (median-IQR)	497	16.6	14.6-18.3	1	0.95-1.03	0.6
Vomiting	**189**	**32**	**16.9**	**0.55**	**0.32-0.92**	**0.02**
Joint pain	10	3	30	1.54	0.25-6.88	0.46
Skin or facial flush	134	35	26.1	1.38	0.84-2.24	0.18
Generalized body rash	7	3	42.9	2.71	0.39-16.29	0.18
Abdominal pain	68	20	29.4	1.59	0.85-2.9	0.12
Watery stools	17	4	23.5	1.1	0.26-3.65	0.77
Spontaneous bleeding	**181**	**56**	**30.9**	**2.22**	**1.41-3.5**	**<0.001**
Skin	164	55	33.5			
GI	4	0	0			
Vagina	3	1	33.3			
Nose	16	1	6.3			
Abdominal tenderness	**48**	**19**	**39.6**	**2.61**	**1.32-5.06**	**0.003**
Liver enlargement	**8**	**5**	**62.5**	**6.14**	**1.17-40.18**	**0.01**
Lethargy	31	4	12.9	0.51	0.13-1.51	0.27

*Values in **bold** are significant at P <0.05. BMI: body mass index. IQR: inter-quartile range. GI: Gastrointestinal track*

5.3.6. Haematological profile at enrolment

We considered the haematological and liver transaminase results in study participants at the time of enrolment and whether any of these variables were associated with progression to DHF. Box-plots showing differences between DHF group and the DF/OFI group are presented in Figure 5-3. At the time of enrolment, the haematocrit was significant higher in DHF patients relative to the rest of the patient population (DF/OFI group (40% vs. 38%, respectively, P<0.0001, Wilcoxon test). In contrast, platelet and white blood cell counts were significantly lower in DHF patients (Table 5-4). The percentage of patients who had AST or ALT levels greater than 40IU (upper limit of normal range) was also significantly higher in DHF patients than the DF/OFI group (both P<0.0001, Fisher's Exact).

Table 5-4: Hematological profile of patients at enrolment.

Variable	DF/OFI N=388	DHF N=109	P
Haematocrit (%)	38 (36–41)	40 (37–43)	0.001^{+}
Platelet count (x1000 cells/μl)	181 (141.8–239.3)	138 (98–172)	<0.0001
White blood cell (10^3 cells/μl)	5 (3.6–7.5)	3.7 (2.4–4.6)	<0.0001
Neutrophil (%)	66 (53–75)	66 (54–73)	0.9
Lymphocyte (%)	20 (14–31)	21 (13–29)	0.8
AST >40U/L N (%)	201 (51.8%)	88 (80.7%)	<0.0001*
ALT >40U/L N (%)	69 (17.8%)	54 (49.5%)	<0.0001*

Values are shown in median (IQR) or n (% total). $^{+}$ *adjusted for age. * Fisher's Exact test.*

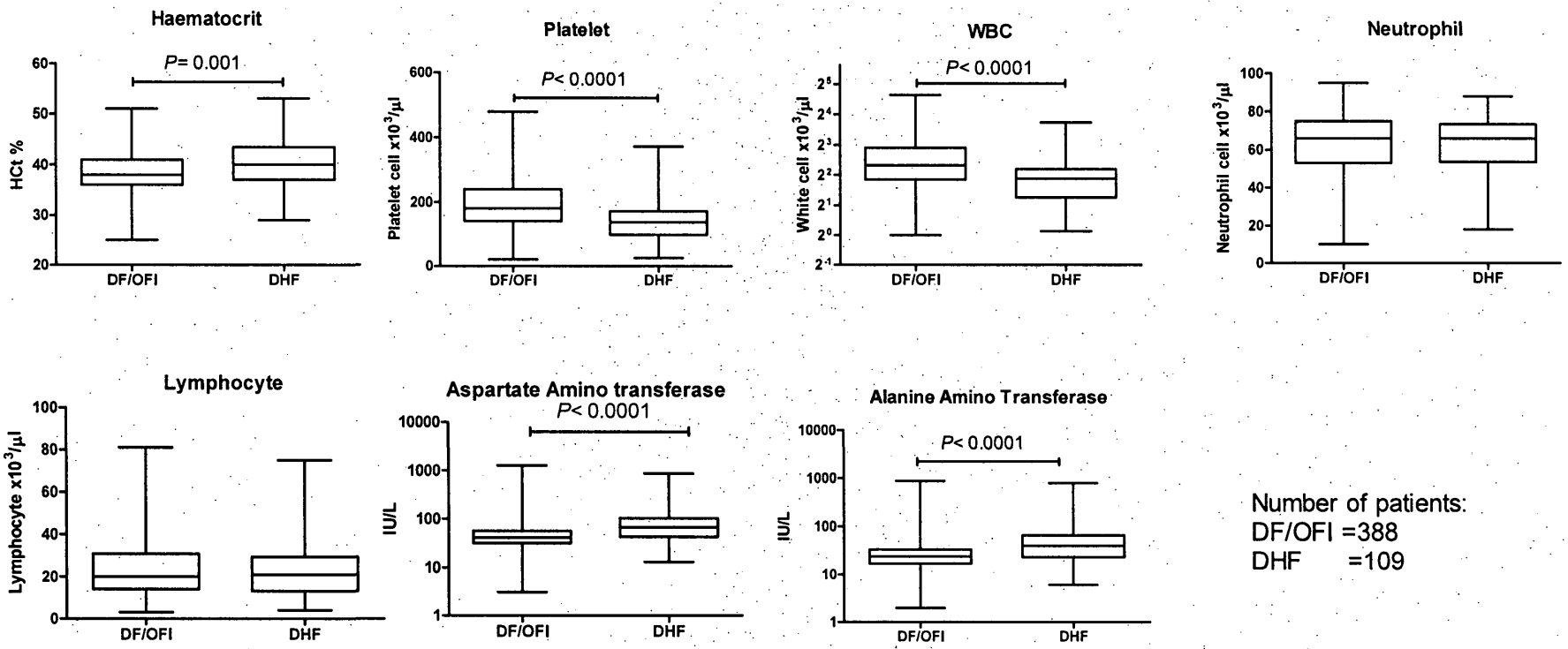


Figure 5-3: Differences in haematological profile between DHF and DF/OFI at enrolment.

Box and whisker plots of haematological parameters at enrolment represent median, 25th and 75th percentile, minimum and maximum of the data classifying by disease severity.

5.3.7. Prognostic models

Multivariate analyses were performed to construct prognostic models that might be useful for the identification of patients who progress to DHF. We used logistic regression for model development and report full models and selected models with stepwise model selection according to the Bayesian information criteria (BIC). Models were developed upon clinical and/or haematological features of patients at enrolment.

Logistic regression models based on clinical features at enrolment to predict DHF

The full clinical model includes age, gender, vomiting, spontaneous bleeding, abdominal tenderness and liver enlargement covariates seen at enrolment. A number of variables were excluded due to non-significant ($p>0.05$) results in univariate analysis. While the clinical model selected by BIC includes only age and bleeding signs at enrolment (Table 5-5). The performances of the models in predicting DHF cases at enrolment are shown by ROC curves in Figure 5-4. The full model had higher area under ROC curve than the selected model (AUROC=0.68 vs. 0.64, respectively)

Table 5-5: Prognostic models based on clinical symptoms and signs at enrolment.

Covariate	Full model			Model selected by BIC		
	OR	95% CI	P	OR	95% CI	P
Intercept*	0.06	0.02 – 0.15	<0.0001	0.1	0.05 – 0.17	<0.0001
Gender	1.56	1 – 2.45	0.051	-	-	-
Age	1.08	1.02 – 1.14	0.007	1.08	1.03 – 1.14	0.004
Vomiting	0.54	0.31 – 0.92	0.025	-	-	-
Spontaneous bleeding	1.91	1.22 – 3	0.005	2.08	1.34 – 3.21	0.001
Abdominal tenderness	2.21	1.09 – 4.47	0.027	-	-	-
Liver enlargment	4.89	0.98 – 24.37	0.053	-	-	-

** Intercept corresponds to a one year old male patient who didn't have symptoms/signs of vomiting, bleeding, abdominal tenderness and liver enlargement.*

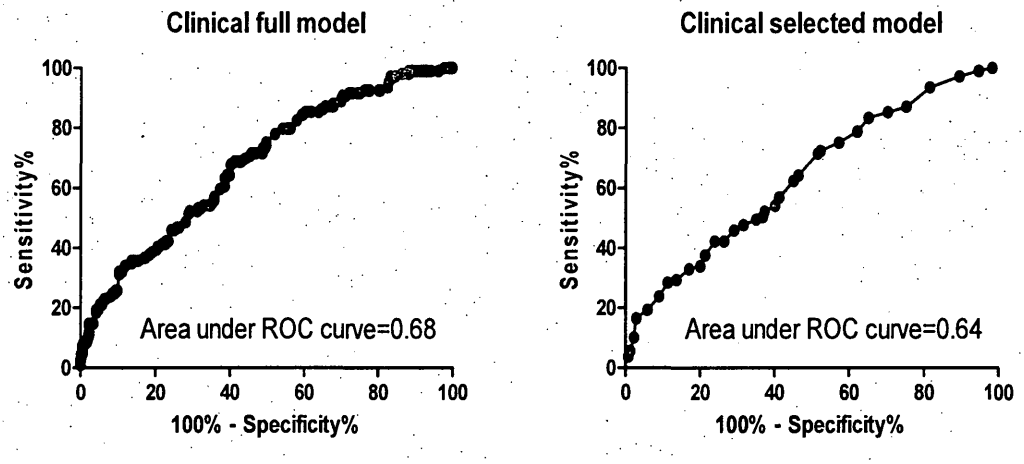


Figure 5-4: Receiver-operator characteristic (ROC) curves for prognosis of DHF using clinical symptoms and signs at enrolment.

The clinical full model derived from age, gender, vomiting, spontaneous bleeding, abdominal tenderness and liver enlargement covariates seen at enrolment and the clinical selected model includes only age and bleeding signs at enrolemnt.

Logistic regression models based on haematological profile at enrolment to predict DHF

Similarly with the clinical models, we constructed a full model and a selected model based on BIC using haemotological parameters at enrolment. The haematological full model includes gender, age, haematocrit, platelet, white blood cell count, AST and ALT covariates, the excluded covariates were percentages of lymphocyte and monocyte because of their non-significant ($p>0.05$) results in univariate analysis. The numbers of covariates that were excluded by BIC were age, gender and AST>40 (Table 5-6). The ROC curves of those two models are presented in Figure 5-5. The area under ROC curve of the full model is similar to that of the selected model (AUROC=0.81 vs. 0.80, respectively).

Table 5-6: Prognostic models based on haematological profile at enrolment.

	Full model			Model selected by BIC		
Covariate	OR	95% CI	P	OR	95% CI	P
Intercept*	0.09	0.02 – 0.39	0.002	0.67	0.29 – 1.53	0.34
Age	1.06	1 – 1.13	0.054	-	-	-
Gender	1.67	1.01 – 2.74	0.042	-	-	-
Haematocrit (by +10%)	1.88	1.02 – 3.48	0.043	2.11	1.19 – 3.76	0.011
Platelet (by +100 x10^3 cells/µl)	0.55	0.35 – 0.86	0.009	0.49	0.32 – 0.76	0.001
WBC (by 2-fold increase) x10^3cells/µl	0.3	0.14 – 0.63	0.002	0.24	0.11 – 0.49	<0.0001
AST>40 U/L	2	1.1 – 3.64	0.022	-	-	-
ALT>40 U/L	3.33	1.95 – 5.68	<0.0001	3.55	2.17 – 5.83	<0.0001

*WBC: white blood cell count. * Intercept corresponds to a one year old male patient with haematocrit of 40%, 200 x10^3 platelet cells/µl, 1000 white blood cells/ µl and AST and ALT<40 U/L. The full model includes covariates such as age, gender, haematocrit, platelet, WBC, AST and ALT greater than 40 UlL. The selected model includes covariates such as haematocrit, platelett, WBC and ALT greater than 40.*

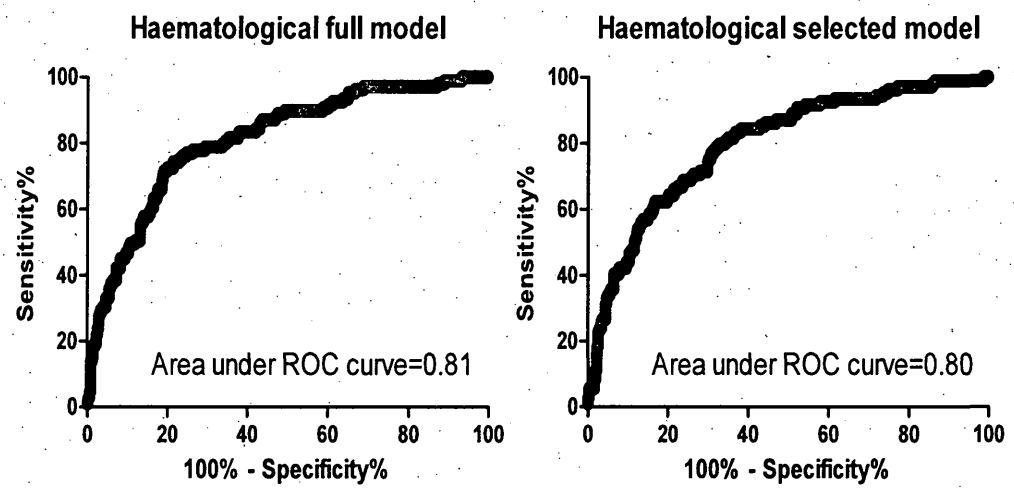


Figure 5-5: Receiver-operator characteristic (ROC) curves for prognosis of DHF using haematological profile at enrolment.

Comparison to other models

Since several models may explain the data similarly well and there is no guarantee that any model selection criteria selects the true model, we also analyzed the data using Bayesian model averaging and reported all models (Appendix 2 and 3 show models from the clinical and haematological parameters, respectively). The best models using Bayesian model averaging method were similarly plausible as the models selected by BIC. We also constructed other models with clinical and haematological parameters altogether (Appendix 4). However, model selection by BIC based on clinical and haematological covariates led to exactly the same covariates as the haematological selected model. This indicated that further adding other clinical to haematological covariates doesn't improve prediction.

Furthermore, we also constructed tree models based on clinical or haematological parameters (Appendix 5 and 6, respectively) then analyzed them. However, the performance of the tree model based on clinical covariates was similar to that of the clinical logistic model selected by BIC while the tree model based on haematological covariates was difficult to interpret with 6 covariates and 12 terminal nodes. Thus, the haematological logistic model selected by BIC was the simplest but

had the best area under ROC curve (AUROC=0.8). For practical utility, we built a nomogram from the haeamtological selected models (Figure 5-6 with an instruction on the footnote) corresponding with probability of DHF. The smaller the probability of DHF is the higher sensitivity and the lower specificity are gained (i.e. at probability of 0.8, sensitivity and specificity are 2.8% and 100%, respectively; at probability of 0.1, sensitivity and specificity are 91% and 43%, respectively).

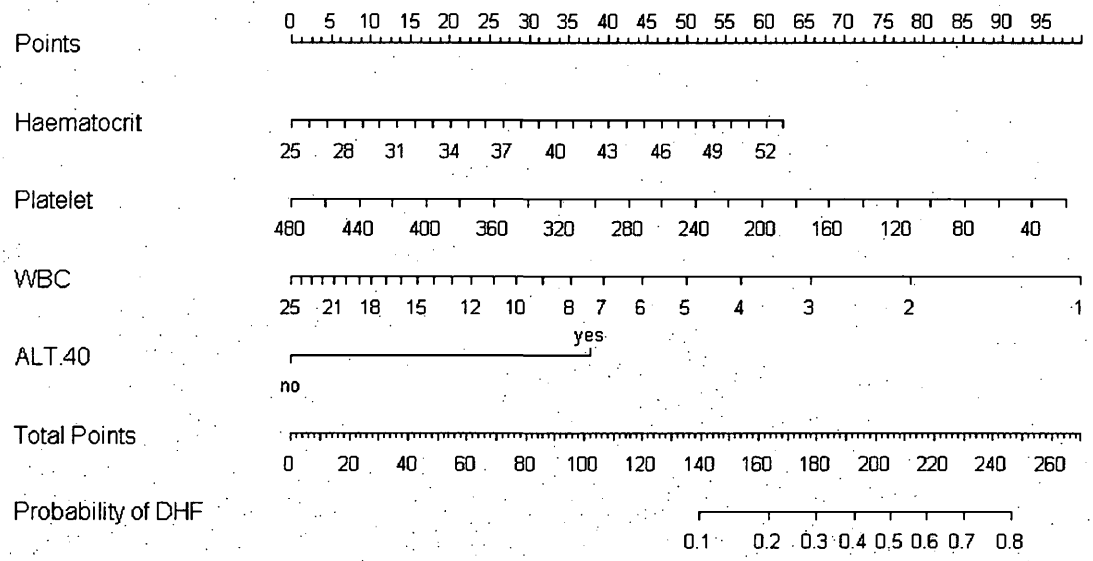


Figure 5-6: Nomogram for prognosis of DHF using haemotological profile at enrolment.

ALT.40 means ALT level>40 U/L. Platelet and WBC are in ($x10^3$ cells/µl) unit. Add up points which correspond to values of the parameters of an individual then from the total points we have, we can know the probability of the individual progress to DHF. For example, a patient who is clinically suspected dengue within the 3 days of his illness presents with 46% haematocrit, 160x10^3 platelet cells/µl, 5 x10^3 WBC and ALT 50 U/L. Thus, he has a total of 200 points (from 46+67+50+37). The probability of the patient progressing to DHF is 0.45.

5.3.8. Performances of the models for prognosis of DHF

The performances of the clinical and haematological selected models are summarized in Table 5-7. A probability of greater than 0.2 (20%) was chosen as the cut-off value for the predictive models. Of note, as clinically defendable thresholds, this cut-off implies that we implicitly qualified the benefit of a correct DHF classification as 4-times greater than the harm of a false DHF classification. Resubstitution of the original data set into the prognostic model that was generated from clinical parameters had 64% sensitivity and 54% specificity. While the model generated from haematological parameters had 79% sensitivity and 68% specificity. The sensitivity, specificity, NPV and percentage of correctly classified patients of the model constructed from haematological parameters were significantly higher than that of the model constructed from clinical parameters ($p<0.05$) while, the PPV was similar between the two models.

Table 5-7: Performance of the models for the prognosis of DHF at the cut-off 0.2

	The clinical selected model		The haematological selected model		P^+
	Apparent*	Optimism-corrected**	Apparent	Optimism-corrected**	
Sensitivity	70/109 (0.64)	0.59	86/109 (0.79)	0.76	0.02
Specificity	208/388 (0.54)	0.53	262/388 (0.68)	0.67	<0.0001
PPV	70/180 (0.28)	0.26	86/212 (0.41)	0.39	0.76
NPV	208/247 (0.84)	0.82	262/285 (0.92)	0.91	0.007
Accuracy	278/497 (0.56)	0.54	348/497 (0.7)	0.69	<0.0001
AUROC	0.64	0.60	0.8	0.78	-

*AUROC: Area under ROC curve, PPV and NPV are positive and negative predictive value, respectively. Accuracy of the test was calculated by * resubstitution or by ** bootstrap validation (repeated 100 times) using the original data set that generated the model to overcome the optimism from resubstitution. + Comparison between apparent values of the two models using Fisher's Exact test.*

The results show that the haematological was the best model for prognosis of DHF using haematological profile at enrolment. It was simplest and had the best performance of all models that were generated.

5.3.9. Plasma viraemia

A summary of plasma viraemia characteristics between DF and DHF are shown in Table 5-8 and 5-9 by serotype and serological status, with p-values for all pre-defined group comparisons in the footnote. The kinetic changes in viraemia were plotted by day after fever onset (Figure 5-7). Viraemia AUCs and viraemia concentration at enrolment were highly correlated with each other (partial correlation between log-values after controlling for gender, age, day of illness, serotype and serological status, r=0.93, p<0.0001).

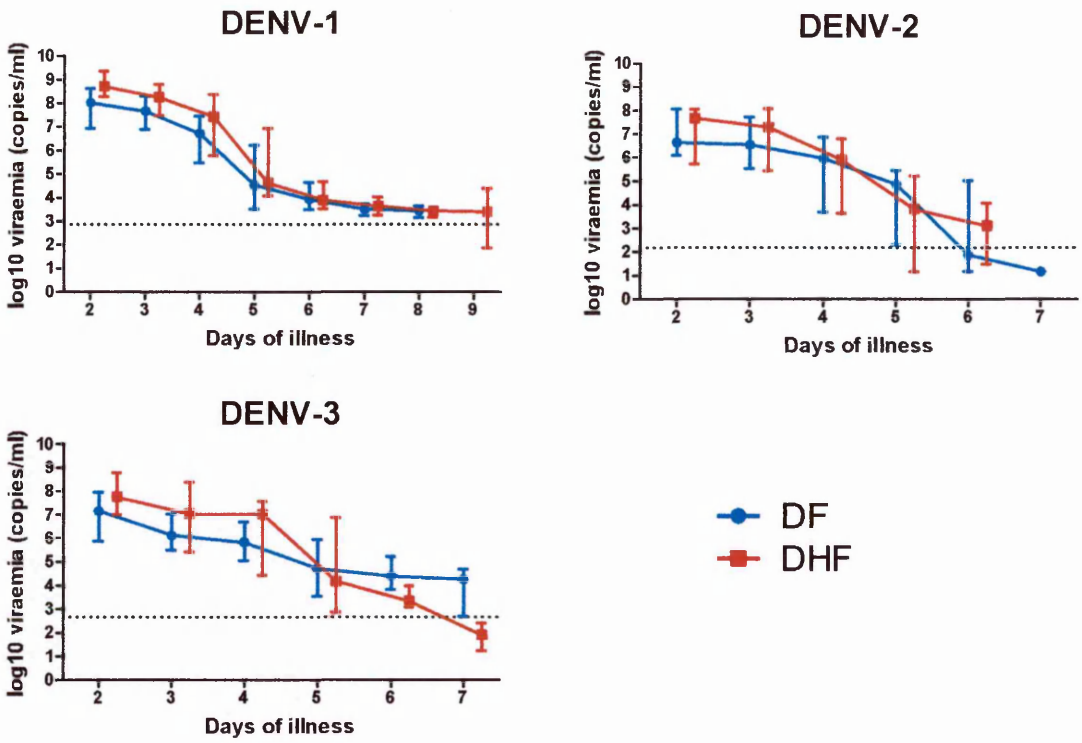


Figure 5-7: Viraemia kinetics in 263 children with dengue by serotype and disease severity over illness day.

Graphs show means and IQRs of viraemia over day of illness. Missing data was excluded in the analysis.

Children with DHF had almost one log10-copies/ml higher plasma viraemia at enrolment and AUCs than children with DF (comparisons were adjusted for serotype, serological status and day of illness, p=0.0009 and p=0.03, respectively) (Table 5-8). However, time to viral clearance was borderline statistically longer in DHF cases than children with DF (p=0.051). The percentage of patients with a viraemia that was till detected in the plasma sample collected at discharge was similar between two groups, 37.3% (66/177) in DF group compared with 38.6% (34/88) in DHF group (p=0.9, Chi-square test). When we stratified patients by serotype and disease severity, the comparisons revealed that viraemia levels at enrolment in DHF patients were not significantly higher than DF patients infected with the same serotype, DENV-2 or DENV-3 (both adjusted p values>0.1). In DENV-1 infected patients however, viraemia levels at enrolment were higher in patients with DHF than DF (adjusted p=0.02). The comparisons between serotypes revealed that viraemia levels were significantly higher (~1 log10 higher) in patients with DENV-1 infection compared with the other serotypes (all p<0.1 for enrolment measurements, all p<0.05 for AUCs, footnote of Table 5-8) and DENV-1 had a significant longer viral clearance time than other serotypes (all p<0.001).

Table 5-8: Summary and comparison of plasma viraemia levels by DENV serotype and disease severity.

	Total		P+	DENV-1		DENV-2		DENV-3	
	DF	DHF		DF	DHF	DF	DHF	DF	DHF
N	177*	88		109	59	22	15	44	14
At enrolment [log10-copies/ml]	7.38 (6.29 - 8.19)	8.2 (7.43 - 8.9)	0.0009	7.87 (6.91 - 8.49)	8.38 (7.87 - 9.05)	6.78 (5.95 - 8.08)	7.52 (5.75 - 8.07)	6.27 (5.7 - 7.43)	7.58 (6.68 - 9.02)
AUC [log10-copies/ml]	7.52 (6.44 - 8.45)	8.14 (7.37 - 8.92)	0.0008	7.89 (7.05 - 8.63)	8.4 (7.74 - 9.11)	6.95 (6. - 7.94)	7.57 (6.03 - 8.25)	6.32 (5.48 - 7.71)	7.39 (6.38 - 8.83)
Time to viral Clearance [days]**	7 (6 - 8)	7 (6 - 8)	0.051	8 (6 - NA)	8 (7 - 9)	6 (5 - 6)	5 (5 - 6)	5 (5 - 6)	6 (4 - 7)

*Numbers are reported as median and IQR. * include 2 children with DENV-4 infection. ** Kaplan-Meier estimates; viral clearance was documented in 165/265 (62.3%) children. + Comparisons between DF and DHF were adjusted for age, gender, day of illness, serotype and serological status. Other comparisons of serotypes were adjusted for age, gender, day of illness and serological status, p-values adjusted for multiple comparisons (D1.DF=DENV-1, DF patients, etc.):*

Log10 copies/ml at enrolment:

D1.DF-D1.DHF p=0.02; D1.DF - D2.DF p=0.096; D1.DF - D3.DF p< 0.001

D1.DHF - D2.DHF p< 0.001; D1.DHF - D3.DHF p= 0.069

D3.DF-D3.DHF p=0.088

Log10 AUC:

D1.DF – D1.DHF p=0.076; D1.DF - D2.DF p= 0.026; D1.DF - D3.DF p< 0.001

D1.DHF - D2.DHF p<0.001; D1.DHF - D3.DHF p= 0.042

Time to viral clearance:

D1.DF - D2.DF; D1.DF - D3.DF all p< 0.001

D1.DHF - D2.DHF; D1.DHF - D3.DHF all p< 0.001

All other adjusted p>0.1

When we stratified patients by serological status and disease severity (Table 5-9), viraemia levels detected in DHF patients with secondary infection were significantly about 1 log10-copies/ml higher than in DF patients with secondary infection (adjusted p=0.003 for viraemia at enrolment and adjusted p=0.04 for AUCs) and the viral clearance time was also longer in DHF group with secondary infections than DF group with secondary infection (p=0.045). Among primary infections, the results suggest that higher plasma viraemia (at enrolment and AUC) occurs in DHF patients compared to DF patients, but the number of DHF patient was small (n=8) and no statistically significant differences were observed. Besides, most of the comparisons between serological statuses revealed that enrolment viraemia, AUCs and viral clearance time weren't significant lower in secondary than primary infections. However, DF patients with secondary infections had a significant shorter viral clearance time compared with those of DF patients with primary infections (median time=6 vs. 8, p=0.006, comparisons adjusted for age, gender, day of illness and serotype).

In summary, viraemia levels were higher in DHF than DF patients (in term of levels at enrolment and AUCs). The differences between DHF and DF were observed in the group of patients with secondary infection. Viraemia levels at enrolment, AUCs and viral clearance time measured in DENV-1 infected patients were higher than those in DENV-2 or DENV-3 infected patients. Finally, there wasn't any difference between primary and secondary infections in term of viraemia at enrolment and AUCs. However, patients with primary infection had longer viral clearance time than patients with secondary infection.

Table 5-9: Summary and comparison of plasma viraemia levels by serological status and disease severity.

	Total*		DF		DHF	
	Primary	Secondary	Primary	Secondary	Primary	Secondary
N	60	205	52	125	8	80
At enrolment [log10-copies/ml]	7.74 (6.73 – 8.35)	7.69 (6.53 – 8.52)	7.55 (6.51 - 8.25)	7.21 (6.24 - 8.19)	8.48 (7.39 - 9.3)	8.15 (7.43 - 8.85)
AUC [log10-copies/ml]	7.8 (6.73 – 8.73)	7.75 (6.63 – 8.54)	7.68 (6.73 - 8.61)	7.43 (6.27 - 8.38)	9.16 (7.15 - 9.48)	8.1 (7.37 - 8.77)
Time to viral clearance [days]	8 (7-NA)	7 (6 - 8)	8 (7-NA)	6 (5 - 8)	8 (NA)	7 (6 - 8)

NA: not available. Numbers are reported as median and IQR. Comparisons were adjusted for age, gender, day of illness, serotype and disease severity reveal only one significant difference in the clearance time (primary infection had longer viral clearance time than secondary infection, $p<0.001$), Other comparisons were adjusted for age, gender, day of illness and serotype, p-values adjusted for multiple comparisons (P.DF=DF patients with primary infection, S.DF=DF patient with secondary infection etc.):*

***Log10 copies/ml**: S.DF - S.DHF $p=0.003$*

***Log AUC**: S.DF - S.DHF $p=0.04$*

***Time to viral clearance:** P.DF - S.DF $p=0.006$; S.DF - S.DHF $p=0.045$.*

All others adjusted $p>0.1$.

5.3.10. Plasma cytokine

Various pro- and anti-inflammatory cytokines have been implicated in the pathogenesis of DHF. To explore whether there were early differences in cytokine concentrations between DHF and DF patients, and if these could be of prognostic value, we measured concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα in enrolment plasma samples of the patients who had serological confirmed dengue, detectable viraemia and NS1 at enrolment. Table 5-10 summaries characteristics of the patients at enrolment.

Table 5-10: Characteristics of subgroup of patients who had plasma cytokine measurements performed.

Parameters	DF N= 134	DHF N= 66
Age	10 (7 – 13)	12 (7 – 15)
Female	55 (41%)	31 (47%)
DOI at enrolment	3 (2 – 3)	3 (2 – 3)
Fever day at enrolment	-3 (-4 – -2)	-4 (-5 – -2)
Length of hospitalization*	5 (4 – 6)	6 (5 – 7)
Log10 plasma viraemia* (copies/ml)	7.5 (6.4 – 8.2)	8.3 (7.7 – 9)

Numbers are reported as median (IQR) or N (% total). * Comparisons between two group show both $p<0.0001$ (Wilcoxon rank sum test).

At enrolment, the concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα were significantly higher in plasma samples from children with DHF than those in samples from children with DF (all $p<0.05$, comparisons of log-10 values adjusted for age, gender, serological status and day of illness). Of the cytokines measured, only concentrations of IL-5 were not significantly different between DHF and DF patients ($p= 0.063$) (Table 5-11, Figure 5-6). In the multivariable analysis adjusted for age, gender, serological status and day of illness, IL-6 and IL-13 were independently associated with DHF (OR=1.2 and 1.1, $p=0.024$ and 0.014, respectively).

Comparisons between the disease severity among patients with secondary infections (Table 5-11) revealed that IL-6, IL-10, IL-13 and TNFα concentrations at enrolment were significantly lower in DF than DHF patients (log10 pg/ml comparisons, 1.4 vs. 1.6, p=0.04 for IL-6; 1.1 vs. 1.3, p=0.02 for IL-10; 0.93 vs. 1.16, p=0.048 for IL-13 and -0.1 vs. 0.1, p=0.01 for TNFα). IL-1 and IFNγ were also lower in DF than DHF patients with secondary infection, but were of borderline significance (adjusted p=0.07 and 0.09, respectively). Among primary infections, these cytokines at enrolment were also generally lower in DF patients compared with DHF patients, but there were no significant differences due mainly to the small number of the primary infection patients with DHF (6 patients). These results suggested IL-6, IL-10, IL-13 and TNFα might play an important role in pathogenesis of DHF in secondary infections. Interestingly, no statistically significant difference was seen when comparisons between primary and secondary infections were made. This implies that primary or secondary infection didn't effect on cytokine production.

Table 5-11: Plasma cytokine concentrations at enrolment by dengue severity or serological status.

	Total		P	Adjusted P*	Primary		Secondary	
	DF	DHF			DF	DHF	DF	DHF
N	134	66			47	6	87	60
log10-IL1β (pg/ml)	-0.02 (-0.26 - 0.25)	0.15 (-0.07 - 0.39)	0.005	0.016	0.05 (-0.22 - 0.3)	0.31 (-0.1 - 0.51)	-0.12 (-0.27 - 0.23)	0.14 (-0.09 - 0.38)
log10-IL2 (pg/ml)	0.96 (0.67 - 1.33)	1.18 (0.85 - 1.48)	0.026	0.045	1.09 (0.67 - 1.35)	1.29 (0.79 - 1.72)	0.92 (0.62 - 1.31)	1.18 (0.82 - 1.48)
log10-IL4 (pg/ml)	-0.28 (-0.64 - 0.19)	0.1 (-0.48 - 0.37)	0.011	0.013	-0.21 (-0.49 - 0.26)	0.17 (-0.05 - 0.63)	-0.35 (-0.64 - 0.19)	0.06 (-0.49 - 0.36)
log10-IL5 (pg/ml)	0.6 (-0.7 - 0.96)	0.83 (-0.7 - 1.05)	0.095	0.063	0.61 (-0.7 - 1.)	0.51 (0.08 - 1.04)	0.6 (-0.7 - 0.95)	0.84 (-0.7 - 1.08)
log10-IL6 + (pg/ml)	1.41 (1.22 - 1.65)	1.59 (1.42 - 1.74)	0.001	0.006	1.44 (1.27 - 1.59)	1.55 (1.42 - 1.69)	1.4 (1.19 - 1.71)	1.6 (1.42 - 1.75)
log10-IL10 (pg/ml)	1.08 (0.84 - 1.33)	1.29 (1.05 - 1.74)	0.0002	0.002	0.99 (0.84 - 1.22)	1.11 (0.91 - 1.91)	1.12 (0.83 - 1.45)	1.3 (1.05 - 1.66)
log10-IL12p70 (pg/ml)	0.21 (-0.03 - 0.53)	0.45 (0.06 - 0.62)	0.024	0.026	0.31 (0.03 - 0.53)	0.39 (0.02 - 0.69)	0.18 (-0.05 - 0.53)	0.46 (0.07 - 0.62)
log10-IL13 + (pg/ml)	0.96 (0.76 - 1.24)	1.16 (0.87 - 1.4)	0.008	0.013	0.97 (0.72 - 1.28)	1.09 (0.69 - 1.65)	0.93 (0.77 - 1.23)	1.16 (0.88 - 1.39)
log10-IFNg (pg/ml)	1.02 (0.58 - 1.45)	1.24 (1.01 - 1.53)	0.002	0.006	0.93 (0.54 - 1.45)	1.43 (1.09 - 1.96)	1.05 (0.6 - 1.45)	1.23 (0.98 - 1.51)
log10-TNFa (pg/ml)	-0.06 (-0.29 - 0.15)	0.12 (-0.19 - 0.31)	0.004	0.002	0.01 (-0.27 - 0.18)	0.19 (-0.04 - 0.35)	-0.09 (-0.3 - 0.1)	0.12 (-0.19 - 0.31)

*Numbers are reported as median and IQR. + IL-6 and IL-13 were independently associated with DHF at p=0.024 and 0.014, respectively. *P derived from Mann-Whitney test. ** Comparisons were adjusted for age, gender, serological status and day of illness. Other comparisons were adjusted for age, gender and day of illness with p-values adjusted for multiple comparisons (P.DF=DF patients with primary infection, S.DF=DF patient with secondary infection etc.): **IL-1**: S.DF - S.DHF p=0.07; **IL-6**: S.DF - S.DHF p=0.02; **IL-10**: S.DF - S.DHF p=0.02; **IL-13**: S.DF - S.DHF p=0.048; **IFNγ**: S.DF - S.DHF p=0.09; **TNFα**: S.DF - S.DHF p=0.008; All others adjusted p>0.1.*

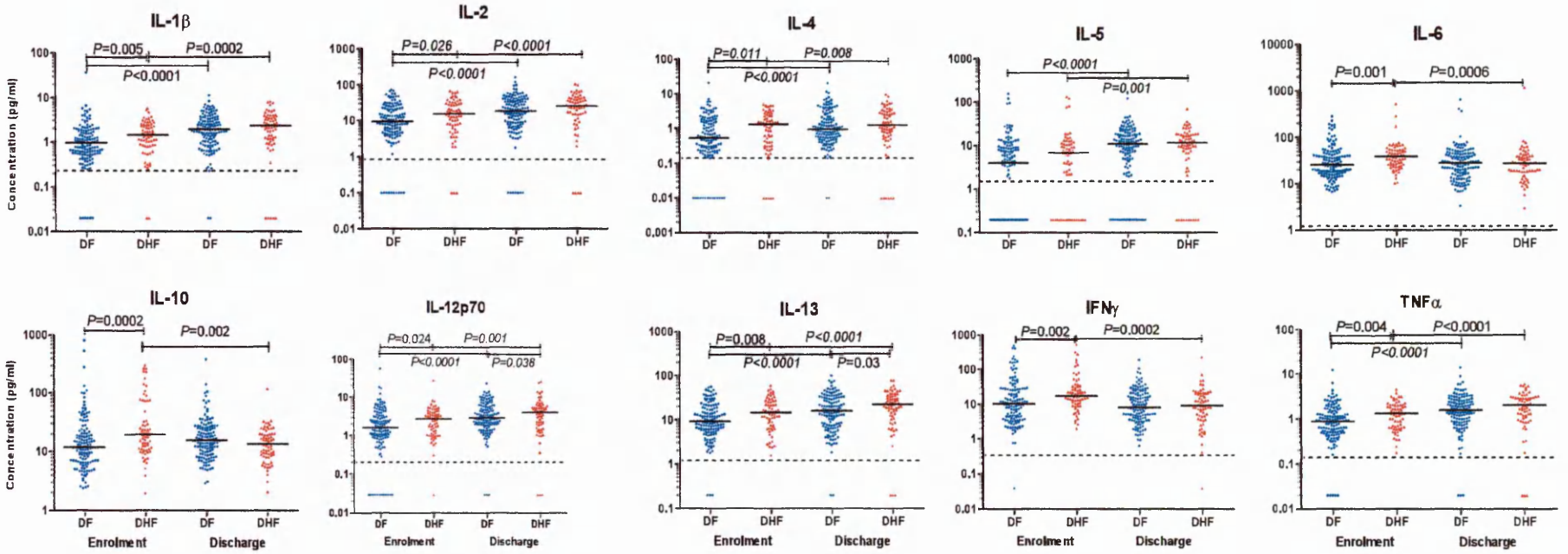


Figure 5-8: Cytokine concentrations in plasma samples at enrolment and discharge by disease severity.

Scatter plots show concentrations of ten cytokines with medians (lines) and limit of detection levels (dash lines) which were measured in plasma samples from children with DF (blue dots, n=134) or DHF (red dots, n=66) at enrolment and discharge. P-values were derived from Mann-Whitney test between patient groups or Wilcoxon paired comparisons between enrolment and discharge samples. In both DF and DHF groups, medians of DOI at enrolment and discharge were 3 days and 8 days, respectively.

Surprisingly, plasma concentrations of IL-1β, IL-4, IL-6, IL-12p70, IL-13, IFNγ and TNFα were significantly higher at the time of discharge from the hospital in children with DHF than those in children with DF; only IL-2 and IL-5 were not significantly different between the two groups at this time point (Figure 5-8). Interestingly, IL-10 concentrations measured at discharge had an inverse trend with that measured at acute phase. Thus, at discharge, IL-10 was lower in plasma sample collected from children with DHF than those from children with DF (median of log10-pg/ml, 1.13 vs. 1.19 pg/ml; p=0.0002, adjusted for age, gender, day of illness and serological status). The results suggested that IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα still play their role in pathogenesis of DHF during convalescence phase.

5.3.11. Correlations between viraemia and cytokine concentrations at enrolment

At enrolment, higher viraemia levels were significantly associated with higher concentrations of IL-1β, IL-2, IL-4, IL-12p70, IL-13 and TNFα (partial correlation controlled for age, gender and day of illness, all $r<0.3$, $p<0.04$). Higher viraemia levels were also significantly correlated with lower concentrations of IL-10 (partial correlation $r= -0.21$, $p=0.004$); while, IL-5, IL-6 and IFNγ had no statistically correlation with viraemia burden (Table 5-12). It shows that the high levels of viraemia stimulated concentration of IL-1β, IL-2, IL-4, IL-12p70, IL-13 and TNFα, but conversely, inhibited concentration of IL-10. Production of IL-5, IL-6 and IFNγ weren't effected by the levels of viraemia.

Table 5-12: Correlations between viraemia levels and cytokine concentration at enrolment.

Cytokine	log10-IL1β (pg/ml)	log10-IL2 (pg/ml)	log10-IL4 (pg/ml)	log10-IL5 (pg/ml)	log10-IL6 (pg/ml)
Correlation, r	0.20	0.25	0.23	0.12	0.07
P (2-tailed)	0.01	0.0007	0.002	0.11	0.37
Cytokine	**log10-IL10 (pg/ml)**	**log10-IL12p70 (pg/ml)**	**log10-IL13 (pg/ml)**	**log10-IFNg (pg/ml)**	**log10-TNFa (pg/ml)**
Correlation, r	-0.21	0.15	0.30	0.02	0.21
P (2-tailed)	0.004	0.04	<0.0001	0.84	0.004

Partial correlation controlled for age, gender, day of illness of the samples.

5.4. Discussion

The ability to identify in the early stages of dengue those patients who will develop clinical complications could significantly influence clinical management and the use of health care resources. In this prospective study of 497 patients with suspected dengue and enrolled within the first three days of illness, we characterized clinical, virological and cytokine profiles in dengue cases and identified early features that were associated with DHF. We developed models to predict patients with DHF that had high specificity but unfortunately also had low sensitivity.

Our study proposed a model that has the ability to make a quite accurate prediction of DHF (AUROC=0.8) in the first few days of illness. It could help physicians immediately pay attention to patients who are at high risk of DHF/DSS and give them suitable care to reduce mortality. Additionally, our understanding of the dynamics of viraemia, host response and clinical complications in dengue is widened; it is likely that the success of novel interventions with anti-viral drugs or corticosteroids to modulate the host pro-inflammatory response will be dependent on early (≤3 days of illness) clinical, or better, laboratory diagnosis.

In the first 3 days of illness, we observed that a higher proportion of DHF cases had vomiting, spontaneous bleeding, abdominal tenderness and liver enlargement. DHF cases also had higher haematocrits, lower platelet counts, more pronounced leucopenia and increased AST or ALT levels. Most of these variables have been shown to distinguish between dengue and patients with other acute febrile

illnesses [69, 227-232]. These results implied that clinical and haematological parameters might be useful for the prognosis of DHF. The logistic models or classification tree models built on these clinical and/or haematological parameters had moderate to adequate performances (AUROCs range from 0.6 – 0.8)

Previous studies have developed classification trees which had sensitivities and specificities for the diagnosis of dengue that ranged from 70%- 96% [228, 233]. Other studies have considered the prognosis of severe dengue and had sensitivities and specificities of 78%, 80% [233], 100%, 46% [227] and 98%, 44%, respectively [182]. The study populations in these publications were adults [227, 228, 233] and were enrolled late in their illness (more than 3 days) [227, 228]. Two of the algorithms have limited routine clinical utility in prediction of DHF [227, 233] because their models were generated using virological or serological data which is not known routinely, while our models identify those with DHF from all cases with clinically suspected dengue.

Our study had a similar study setting with that of Potts's *et. al.* [182] but we had a smaller sample size (497 vs. 1230 patients) and a shorter time in recruiting patients (2 vs. 13 years) thus our study might have fewer confounders due to the changes in technology and/or human resourse (doctors, nurses, etc.). Furthermore, if we use the cut-off value at probability of DHF greater than 0.1 for our models, the sensitivity is better (91%) but the specificity is reduced (43%) which are similar with those of the classification tree from Potts's *et. al.* [182]. Moreover, our model built on logistic regression of haematological covariates had a broad utility in practical clinical prognosis and research because it can have a floating cut-off level, i.e. we can flexibly choose the cut-off level for our own purpose. For example, if we were conducting a trial of a therapeutic drug, where we have to weigh the benefit/risk of the intervention to the patient, we can select patients who have a higher probability of developing DHF (greater than 0.8 for example). Alternatively, for routine clinical care we can opt for high sensitivity (at probability of DHF greater than 0.1 for example) to make sure that we don't miss any DHF cases.

DENV-1 was the dominant serotype in this study and it accounted for most of the primary infections (70.6% of primary infections). The prevalence of DENV-1 in the study population was consistent with virological surveillance data for the southern

20 provinces of Viet Nam, in which DENV-1 was the most common serotype detected during the study period, followed by DENV-2, DENV-3 and DENV-4[18]. After adjustment for multiple testing among different levels of disease severity (DF or DHF) and day of illness, higher levels of viraemia and longer viraemia clearance time were significantly associated with DENV-1 relative to patients with DENV-2 or DENV-3 infections. It's possible then that DENV-1 viruses are intrinsically more virulent than DENV-2 or DENV-3 viruses, although all four serotypes can clearly cause severe dengue.

Viraemia levels have previously been shown to be associated with severe disease, in DENV-2 and DENV-3, but in smaller datasets [97, 124-126]. With the advantage of our large data set collected early in the illness phase and an analysis that adjusted for serotype, serological status and day of illness, we confirmed that viraemia levels were significantly greater in DHF patients than in DF patients at early timepoints as well as during the dengue illness. This is an important justification for the development and test of antiviral drugs as an early rapid reduction in the viral burden *in vivo* which might help alleviate symptoms and prevent the development of severe complications.

We did not find any significant differences in viraemia between primary and secondary infection, with the only exception of the viral clearance time which was shorter in the secondary infections among DF patients. This finding agrees with Murgue's study [126], but contrasts with other studies on DENV-1 or DENV-2 infections [97, 220, 234]. The possible reason might be the difference in classifying primary and secondary infections. Vaughn, Yeh and Duyen *et. al.* classified secondary infection based on a clear rise in IgG level by day 5-7, while our study classified primary and secondary infections based on IgM, IgG levels together with day of illness. The other reasons are DENV-2 infections were not common in our study, and DENV-2 primary infections in particular were rare. However, we also did not find any differences between primary and secondary DENV-1 infections and thus, this is somewhat inconsistent with the ADE theory and its role in pathogenesis. It is possibly explained that at the beginning of a secondary infection, ADE boosts viral infection in a very short time so plasma viraemia at the time of study enrolment has

already began decreasing. The other explanation is that we were biased toward children in hospital with clinically apparent dengue.

With regard to cytokine profiles in dengue cases, our study was biased to a subgroup of dengue patients who had high viraemia because all of the patients whose plasma samples we measured cytokines had serologically confirmed dengue, detectable viraemia and NS1 at enrolment. However these patients accounted for 62.9% (200/318) of all laboratory confirmed dengue cases. Furthermore, this subgroup of patients didn't skew the analysis toward DHF patients because the percentage of DHF cases in the subgroup was not significantly different from that in all dengue cases (31.9% vs. 35.9%, p=0.57, Fisher's Exact).

Our findings showed a greater virus burden in DHF patients at enrolment leading to an augmented cascade of immune responses as indicated by plasma concentrations of IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-13 and TNFα in DHF patients compared to DF patients. Significant trends were also seen at discharge. This observation was novel and suggested that the timing of cytokine responses to DENV infection could play an important role in the disease pathogenesis. A previous study showed in the first three days of DENV infection, TNF-α, IL-2, IL-6 and IFN-γ reached high levels [162]. TNF-α and IL-10 were also reported to be elevated in DHF [164-168]. Besides, in the studies from adults with DENV infection, IL-6 [186], IFN-γ, IL-1β, IL-4 and IL-13 were increased in patients with severe dengue [169]. Conflicting with these results, concentrations of IL-2, IL-6 and IFN-γ shown were not different in DF and DHF patients [170-172]. The difference in the literature might be related to the different methods of detection, the different case populations and the timing of specimen collection. Increased levels of IL-10 and other type 2-effectors in DHF were thought to play a role in down-regulating the prior, augmented, release of IFNγ and type 1-effectors [235]. Strikingly, our observation that IL-10 measured at discharge had an inverse trend with that measured at acute phase and was also associated with DHF might suggest the role of IL-10 was different before and after the time of defervescence.

Interestingly, no statistically significant difference in pro-inflammatory cytokine levels was seen between primary and secondary infections. This was inconsistent with results from Prinyadarshini *et. al.* [186], which showed higher levels

of IL-6, IL-8 and IFN-γ in the secondary cases compared to the primary dengue cases. The differences might be because of the differences in the study populations in age and day of illness. In our study, cytokines were measured in plasma of children with dengue within the first three days of illness whilst Priyadarshini *et. al.* measured cytokines between 2-15 days of illness onset in plasma from patients aged from 1- 64 years old.

While the strength of this study was its prospective nature in which we recruited a large number of patients within 72 hours from illness onset, the study also had several weaknesses. Firstly, it was hospital based and therefore does not represent the wider dengue case burden. We also had a small number of patients progressing to DSS (n=15). Secondly, we didn't follow up the patients after discharge; this limitation resulted in 37.7% of patients being still viremic at discharge (as defined by RT-PCR) and that might explain why the time to viral clearance was similar between DHF and DF patients. Thirdly, DENV-1 infections (63.4%) and secondary infections (78.6%) were the majority in our study and therefore we could make few conclusions regarding DENV-3 and DENV-4.

In conclusion, we showed a simple model using early haematological indicators obtained within 72 hours of illness that might be effective in predicting DHF in the clinical setting for pediatric patients with clinically suspected dengue. This might reduce unnecessary hospitalization while optimizing clinical outcome for hospitalized DHF patients. Although, the model needs to be validated in other data sets, the study emphasized the usefulness of haematological profiles at an early stage of illness and is broadly consistent with findings by Potts *et. al.*. Our findings also demonstrated the plasma cytokine profiles in Vietnamese children with dengue. We showed here for the first time that in dengue, different cytokine profiles might be present and vary according to clinical manifestations. Moreover, at the early stage of the disease, there were many biomarkers that could be used to distinguish between DHF and DF patients such as viraemia, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα.

6. Conclusion

The goals of this thesis were to characterize a serological assay for dengue diagnosis and then in prospective studies, identify early clinical, haematological, virological, and immunological factors associated with severe presentations of dengue and then use them to develop predictive algorithms. The specific aims of the study were to:

- Define the diagnostic accuracy of the in-house IgM/IgG ELISA for the diagnosis of acute dengue and the classification of primary or secondary serological status (Chapter 3).

- Determine and evaluate early plasma NS1 cut-off levels for discrimination of DSS from uncomplicated dengue (Chapter 4).

- Develop prognostic algorithms for early identification of severe dengue among patients suspected of having dengue using routine clinical findings and/or routine haematological laboratory markers (Chapter 5).

- Discover early immunological biomarkers of severe dengue (Chapter 5) and strengthen the knowledge of dengue pathogenesis (Chapter 3, 4 and 5).

This study addressed these important questions:

6.1. Why strategies for the early diagnosis and prognosis of severe dengue should be developed?

In the southern 20 provinces of Vietnam, the annual incidence of dengue ranges from 100 to 500 hospitalized dengue cases per 100,000 population [18] and dengue annually represents 4 – 18% of all inpatient diagnoses at three referral hospitals in southern Vietnam [19]. In Vietnam and many other endemic countries, it is not possible to hospitalize all patients with clinically suspected dengue for observation and therefore triage is an important and necessary step. However, the inability to make an early and accurate prediction of which clinically suspected dengue patients might develop complications results in a large number of dengue cases being admitted to hospital who never require any clinical interventions and this patient burden contributes to overcrowding and strain on the health care system. Conversely, some patients are treated as outpatients but who develop complications

that require emergency admission to hospital. The inability to make an early prognosis of severe dengue is also an impediment to novel treatment interventions that target the virus or the host immune response. In patients who are at risk of developing severe complications, the balance of risk versus benefit may favour the use of an intervention. Of note, OUCRU (Vietnam) is conducting randomized controlled trials of short course oral corticosteroids and separately, an anti-viral drug, in dengue patients with fever less than 72hrs (corticosteroids) or less than 48hrs (anti-viral). There is therefore a pipeline of potential therapies for dengue that will be dependent upon making an early diagnosis, and in particular, identifying those patients at the highest risks for complications and who might benefit most from an early treatment intervention. An early prognosis of DHF can assist in patient management by directing clinical attention to the appearance of capillary permeability, for which supportive oral and/or intravenous fluid therapy is recommended in order to prevent circulatory compromise [1, 76, 77] and reduce mortality rate [75]. Furthermore, an accurate dengue diagnosis and prognosis helps to exclude other diagnoses and prevents unnecessary antibiotic usage. A prompt diagnosis of index cases can also facilitate early vector control activities in the community therefore mitigating further transmission.

It is clear that rapid point of care tests would be very useful for diagnosis and prognosis of dengue and severe dengue in outpatient or primary care level facilities. Similarly, they would be useful for intensive care facilities where the differential diagnosis of a patient with shock could include sepsis or other syndromes.

6.2. How did the studies in this thesis strengthen knowledge around diagnosis and prognosis?

Efforts to enhance further understanding of DHF pathogenesis should be priority. The aetiology of DHF appears to be multifactor. These include antibody-dependent enhancement (ADE) [138], cell-mediated pathogenesis [236], cytokine overproduction phenomenon [237], individual genetic background [238], differences among the serotypes [239], levels of virus circulating in the body during the acute phase [97, 240], nutritional status of the infected individual [209, 241]. The results from this thesis support some of them: evidence presented in Chapters 4 and 5 suggest that severity of dengue is dependent upon the relationship between viraemia, antigenic

levels and the host immune response within the first three days of illness and is best predicted by haemotological profile at presentation.

Chapter 3 validated the diagnostic accuracy of in-house ELISA used at the Hospital for Tropical Diseases using a reference panel of serum samples. Chapter 3 also confirmed that antibody profiles elicited by primary infection are different from those caused by secondary DENV infections. This is in line with a previous study done by Gubler *et al* [202]. In primary infections, IgM antibodies develop rapidly with high level while IgG appears slowly afterwards. In secondary infections, high levels of IgG are detectable even in the acute phase whereas IgM levels are low or even absent. Furthermore, chapter 3 proposes an algorithm for discrimination of primary and secondary infection by using the in-house ELISA assays to measure IgM and IgG levels in a single plasma sample collected within 4-14 days of illness. Collectively, these results establish the in-house dengue serology assays as robust diagnostic and research tools.

Chapter 4 supports the hypothesis that the high levels of plasma NS1 within the first few days of illness correlated to high levels of viraemia [127] and high levels of plasma NS1 were associated with progression to DSS [39, 41, 97]. Chapter 4 also shows plasma NS1 cut-off levels within the first 4 days of illness that can predict DSS patients with DENV-1 or DENV-2 infections.

Chapter 5 reveals that increased virus burdens in DHF patients in early phase of illness (within the first three day of illness) correlated with high plasma concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFNγ and TNFα in DHF patients compared to DF patients, the trends were also seen at the time of discharge. The differences in concentration of these cytokines were also observed by others with smaller study populations [162, 164-169] or not long ago, with a larger sample size in both children and adults [186].

Higher haematocrits, lower platelet counts, more pronounced leucopenia and increased AST or ALT levels have been shown to distinguish between dengue and patients with other acute febrile illnesses [69, 227-231]. These laboratory features were also seen frequently in severe dengue [72, 232, 242]. Chapter 5 emphasizes these trends in haematological profile at early illness are connected with development

of DHF; especially, higher haematocrits, lower platelet counts, more pronounced leucopenia and increased ALT levels are independently associated with DHF among clinical and haematological covariates.

The models that are based on early NS1 levels (Chapter 4), clinical and/or haematological or immunological (Chapter 5) profiles would be directly or indirectly used to predict dengue severity with sensitivities and specificities ranging from low to acceptable levels. The models that are based on clinical and haemotological features at enrolment may be used in clinical practice because they are simple and feasible while the models based on NS1 cut-off levels is more difficult for practical utility due to its serotype dependence.

6.3. What are the further directions?

This thesis reveals new basic findings that might assist investigators in establishing novel approaches to combat plasma leakage and haemorrhage in severe dengue. In the future, it is possible that we will use micro-particles and autoloaders to simplify ELISAs (to measure serotype-specific NS1 concentrations, and in parallel, IgM and IgG levels) that can give results within minutes or just a few hours and this may help in diagnosis and prognosis of severe dengue. However, further research is required to evaluate the predictive models using plasma NS1 levels measured within three days of illness. Another study is also needed to evaluate the usefulness of the model using haematological factors in diagnosis and prognosis of DHF for pediatric patients with clinically suspected dengue.

A combination of clinical symptoms, signs, laboratory parameters such as haematological profile, viraemia, NS1, cytokine levels within the first few days of illness and perhaps even host transcriptional profile or genetic background will be crucial in generating "second generation" predictive algorithms capable of identifying severe cases early in the course of the illness. Importantly, any algorithm must be applicable in settings with limited resources such as dengue-endemic countries where these tools are most needed. In terms of biomarkers, detection of multiple biomarkers together might provide the best avenue for accurate diagnosis and prognosis of severe dengue. Newly developed techniques capable of measuring multiple analytes should become important for research in this area. Suitable tests must be cheap, easy and

quick to perform and give an accurate result, ideally without the use of laboratory equipment (lateral flow rapid tests, for example). Since dengue is often a disease of children, an ideal test would require small blood volumes, ideally capillary blood or even other specimens, such as urine or saliva.

7. Appendices

Appendix 1. The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity [35].

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My contributions in the work of this paper were:

- Collecting plasma and doing diagnostic tests (PCR, serology, culture for DENV) to confirm dengue cases
- Isolating DENV from the patients.
- Following up and collecting PBMC of the patients.
- Co-operating in doing memory B-cell immortalization then cloning and screening B cell clones.
- Preparing autologous DENV-infected C6/36 cells for intracellular staining.

Abstract

Dengue virus (DENV) infection and disease is a large and growing global health problem. Antibodies are postulated to confer protection from DENV infection but have also been implicated in enhancing secondary heterotypic infection and increasing risk for severe disease. To understand better the functional repertoire of human antibodies in the context of DENV infection, we studied a large panel of human monoclonal antibodies (mAbs) that were isolated from memory B cells of individuals who recovered from primary or secondary DENV infection. The majority of the isolated antibodies bound to domain I and II of the envelope (E) protein. Notably, these antibodies were broadly cross-reactive against the four DENV serotypes and neutralized poorly, while potently enhancing infection of Fcγ receptor (FcγR)-bearing cells. In contrast, the few mAbs that bound domain III of the E protein showed a more restricted pattern of cross-reactivity and potently neutralized while still modestly enhancing DENV infection. Three E-reactive mAbs that together targeted two epitopes on the four DENV serotypes were engineered with mutations in the Fc region that prevent binding to the FcγR. This mAb cocktail retained full neutralizing activity but failed to enhance infection *in vitro* and protected as post-exposure therapy in a mouse model of Dengue shock syndrome. Our findings indicate that human antibody response to DENV is skewed towards the production of highly cross-reactive antibodies with infection enhancing activity and illustrate the potential feasibility of an antibody based therapeutic to control severe dengue in humans.

Introduction

Dengue virus (DENV) is a mosquito-borne *Flavivirus* responsible for ~50 million human infections annually, including 500,000 hospitalizations and 20,000 deaths, with an economic burden rivalling that of malaria. A primary infection is believed to provide effective, durable and possibly life-long protection against re-infection with the same serotype, but only short-term protection against other serotypes (1, 2). Classical epidemiologic studies suggested that immunity to one of the four DENV serotypes can increase disease severity upon subsequent challenge with a different serotype leading, in some cases, to severe dengue, a disease characterized by plasma leakage and hemorrhagic manifestations (3). Poorly neutralizing cross-reactive antibodies raised in response to a previous serotype are believed to contribute to pathogenesis of severe dengue by promoting virus entry via Fcγ receptors (FcγR) and infection of myeloid cells (4, 5) leading to antibody-dependent enhancement (ADE) of infection. The role of antibodies in severe dengue is supported by epidemiological studies showing that infants with waning levels of maternal antibodies (age 6-9 months) are most vulnerable to severe DENV disease (6, 7), and that serum from these infants enhance DENV infection *in vitro* (8-10). The difficulty of balancing immunity to the four serotypes and minimizing incomplete response and the risk of ADE are major hurdles in the development of a tetravalent vaccine against DENV (11).

The 10.7 Kb RNA genome of DENV encodes three structural proteins, the capsid protein (C), a membrane-associated protein (prM), and an envelope protein (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The E protein is structurally conserved among flaviviruses and consists of three distinct

domains. Domain I (DI) participates in the conformational changes required for viral entry and nucleocapsid escape from the endosomal compartment, domain II (DII) contains the fusion loop, and domain III (DIII) has been suggested to bind cellular receptors (12, 13). Partially mature virions also express varying levels of prM protein on their surface, which is normally cleaved by a furin-like cellular protease to generate the mature virion (14).

The most potent neutralizing antibodies against DENV, or other flaviviruses such as West Nile Virus (WNV), bind to DIII and are effective as passive prophylaxis or therapy in rodents (15-21). The role of antibodies to DI/II is less clear as they tend to be more cross-reactive and less potent in neutralization (22-24). Antibodies to prM generally have poor neutralizing and enhancing activity (25, 26), although recent studies suggest that some anti-prM mAbs can augment infectivity of poorly infectious immature virions (27). Antibodies against NS1, a secreted non-structural glycoprotein that is absent from the virion but expressed on the cell surface, can also protect against infection *in vivo*, through FcγR-dependent and -independent mechanisms (28, 29) or possibly contribute to pathogenesis (30, 31).

Our current knowledge on the human antibody response to DENV is based exclusively on serological studies. In this study we used an improved method of memory B cell immortalization (32) combined with a broad screening approach to isolate a large panel of DENV-reactive mAbs. Most mAbs bound to E protein DI/II or prM and were broadly cross-reactive with the four DENV serotypes. These mAbs neutralized poorly, while potently enhancing infection of $Fc\gamma R^+$ target cells. The few mAbs that bound to DIII of the E protein, showed a more restricted pattern of cross-reactivity and potently neutralized, while still modestly enhancing DENV infection. Based on an improved understanding of the functional humoral response against DENV, we produced a cocktail of three variant recombinant mAbs that neutralized all four DENV serotypes without causing immune enhancement *in vitro* and *in vivo*.

Results

High frequency of DENV-specific memory B cells in immune donors

IgG^+ memory B cells were isolated from frozen PBMC of donors who were diagnosed with either a primary (Donor 76 and Donor 7) or a secondary (Donor 12 and Donor 92) DENV infection and were immortalized with EBV and CpG in multiple replicate wells (from 960 to 2,844), as previously described (32). Culture supernatants of immortalized B cells were collected after two weeks and analyzed using different screening assays (**Table 1**). Supernatants of Donor 76, (primary DENV3) and Donor 12 (secondary DENV1) were screened for their capacity to stain fixed and permeabilized C6/36 mosquito cells infected with DENV isolated from the same donors. For Donors 92 (secondary DENV2) and Donor 7 (primary DENV4) the autologous isolates were not available and therefore the screening was performed by ELISA using as antigen lysates of DENV1, 2, 3, and 4 infected cells (Donor 92) or recombinant E protein from heterologous DENV4 (Donor 7). The frequency of cultures containing specific antibodies ranged from 2.8% to 45% (**Table 1**), depending on the donor and the screening assay used. From these values, by correcting for the efficiency of immortalization, we estimated the frequency of DENV-reactive cells ranging from 1.5% (Donor 7) to 16% (Donor 76) of IgG^+ memory B cells. These findings indicate that in DENV immune donors maintain a very large pool of memory B cells even several months after infection.

Isolation and characterization of human DENV-specific mAbs

A panel of fifty B cell clones were isolated by limiting dilution from independent cultures and the mAbs produced were characterized using binding and functional assays.

All of the mAbs efficiently stained Vero cells infected with at least one of the four DENV serotypes and generally showed distinct patterns of cross-reactivity (**Fig. 1** and **Table S1**). Thirty-one mAbs bound DENV E protein, as assessed by ELISA or Western blot (**Table 2**). Of these, 19 (mostly from Donor 92) cross-reacted with E protein from all four DENV serotypes. Staining of yeast displaying different DENV E protein domains on their surface revealed that these broadly cross-reactive mAbs were specific for DI/II (**Table 2**). Three additional DI/II-directed mAbs from Donor 76 were specific for DENV3 and one E-reactive mAb from Donor 7 was specific for DENV4. Out of 31 mAbs, only 3 recognized DIII of E protein; mAbs DV55.1 and DV87.1 cross-reacted with DENV1, DENV2 and DENV3, whereas mAb DV63.1 cross-reacted with DENV1 and DENV3. Finally, 4 mAbs (DV66.1, DV61.2, DV57.4 and DV77.5), which were selected for binding to DENV-infected cells (**Table S1**), did not bind to recombinant E proteins in ELISA but bound to E protein in Western blot (**Fig. 2A**).

Some of the broadly reactive E-specific mAbs isolated from Donor 92 were analyzed for reactivity against West Nile virus (WNV). Seven mAbs stained yeast cells expressing DI/II of WNV E protein (**Table 3**). Using site-directed mutagenesis (22, 23), we localized binding of three mAbs to the fusion loop peptide at the tip of DII (W101R, G106E) and of a fourth mAb to the W233F residue on DII. The binding of the other three cross-reactive mAbs was not affected by these mutations, indicating that they map to DI/II but outside of the fusion loop region.

Next, we analyzed the capacity of E-reactive mAbs to neutralize or enhance infection by four candidate DENV vaccine strains using flow cytometry assays with Vero and K562 cells, respectively (33, 34). The DIII-specific mAbs DV55.1, DV63.1 and

DV87.1 potently neutralized DENV infection of Vero cells, with EC_{50} values as low as 4 ng/ml, whereas DI/II-specific mAbs showed in general a 10-50-fold lower potency (**Fig 3A and Table 2**). In contrast, the E-reactive mAbs that bound E protein only in Western blot were not neutralizing (**Table 2**), a finding that is consistent with the possibility that the epitopes recognized are not accessible on intact virions. Consistent with what is known about stochiometric relationship between neutralization and enhancement (35), all mAbs that neutralized also enhanced infection over a given range of concentrations (**Fig. 3B** and **Table 2**).

In summary, analysis of the memory B cell repertoire of DENV-infected donors revealed a high frequency of anti-E protein antibodies that were skewed away from DIII-specific neutralizing epitopes and towards cross-reactive determinants with inherently less inhibitory potential.

Human monoclonal antibodies to prM and to non-structural proteins

We next characterized eighteen mAbs that stained DENV-infected cells but did not recognize E protein. Six mAbs bound prM in Western blots (**Fig. 2A**). With the single exception of mAb DV52.1, all the prM-specific mAbs were broadly cross-reactive since they stained cells infected with all four DENV serotypes (**Table S1**). Surprisingly, four of these mAbs also stained a band corresponding to the E protein, and two of them also an additional band of higher molecular weight. The reactivity of mAbs with both prM and E suggests a quaternary epitope with shared sites on the heterodimeric prM-E protein; a similar finding has been reported for mouse antibodies (26, 36). All prM-reactive mAbs

enhanced DENV infection over a broad range of concentrations, while some neutralized infection, although with low potency (**Table 4**).

Eleven mAbs were mapped to NS1 or NS3 protein, as determined by ELISA with recombinant proteins and Western blot (**Fig. 2B** and **Table 5**). Five NS1-specific mAbs cross-reacted broadly with all DENV serotypes, while the remaining two mAbs were specific for DENV1 or DENV3 NS1 (**Table S1**). The four NS3-specific mAbs were all DENV3-specific.

Finally, only one mAb (DV86.2) recognized the capsid protein. Another mAb (DV 34.4) was not assigned to a specific protein, although it showed high neutralizing activity against DENV2 and, to a lower extent, DENV4 (**Table 4**). This mAb likely recognizes a conformationally-sensitive epitope on E (e.g., hinge or dimer interfaces) as was observed recently with two human mAbs against WNV (37).

A broadly neutralizing antibody cocktail lacking enhancing activity

Given the availability of human anti-DENV mAbs, we set out to develop a candidate antibody-based therapy for dengue consisting of a "cocktail" of neutralizing mAbs. Ideally this cocktail should: i) neutralize all DENV serotypes, ii) target at least two non overlapping epitopes on each virus in order to minimize the selection of escape mutants, and iii) fail to enhance infection. We therefore selected two mAbs with potent and complementary neutralizing activity: DV87.1 specific for DIII of DENV1, 2 and 3 and DV22.3 specific for E protein of DENV4 (**Table 2**). As a complement we selected a DI/II-specific mAb (DV82.11) that neutralized with comparable efficiency all four DENV serotypes. Cross-competition experiments were consistent with the notion that the

target epitopes recognized by the three mAbs did not overlap (**Fig. S1**). Thus, a cocktail of DV87.1, DV22.3 and DV82.11 is expected to neutralize all DENV serotypes by targeting on each virus two distinct sites, thus minimizing the chance of selecting escape mutants.

We generated variants of DV87.1, DV22.3 and DV82.11 in which leucine residues at positions 1.3 and 1.2 of CH2 domain (according to the IMGT unique numbering for C-domain) were substituted with alanine residues. This modification, also known as "LALA" mutation, abolishes antibody binding to both FcγRI and FcγRII (38). The variant and unmodified recombinant mAbs were expressed in HEK293T cells and the purified mAbs were compared for their capacity to neutralize and enhance infection by the four DENV serotypes. As shown in **Fig. 4**, LALA variants retained the same neutralizing activity of modified mAbs, but were completely devoid of enhancing activity. In addition, the LALA cocktail, even at very low concentrations (DV82.11 = 2μg/ml; DV87.1 = DV22.3 = 0.2μg/ml), blocked ADE induced by enhancing mAbs or immune serum (**Fig. 5**).

To determine whether the combination of LALA mAbs might achieve increased neutralization we used a variable ratio approach (39). The concentration of one mAb was varied in the presence of a fixed amount of a second mAb (added at a weakly neutralizing concentration) and the titration curves of the first mAb alone or in combination were compared. The EC_{90} values shown in **Table S2** indicate a two- to four-fold increase in neutralization potency consistent with an additive effect.

Taken together, the above results suggest that a cocktail of three variant mAbs targeting different epitopes on each virus can efficiently neutralize DENV infection while avoiding and actually preventing DENV infection enhancement.

LALA variant mAbs do not enhance DENV infection and show therapeutic efficacy *in vivo*

To assess the ability of LALA variants to neutralize DENV *in vivo*, we utilized a recently-described mouse model of DENV ADE where sub-lethal infection is enhanced by passive transfer of anti-DENV antibodies leading to lethal disease characterized by plasma leakage, elevated serum cytokines and thrombocytopenia, all features associated with severe dengue in humans (40). Unmodified or LALA variant mAbs were transferred into AG129 mice 24 hours prior to a sub-lethal infection with DENV2 strain D2S10. While mice pretreated with 1 or 5 μg unmodified mAbs DV82.11 and DV87.1 showed enhanced lethal DENV infection, mice receiving the same amounts of LALA variants did not succumb to infection (**Fig. 6A**; $p<0.0.5$, both DV82.11 and DV87.1, n = 3/group) or show signs of illness. In contrast, mice receiving 5 μg of the mouse mAb 4G2 (pan-flavivirus, E DI/II-specific), succumbed to an enhanced, lethal D2S10 infection as observed previously (40), whereas 5 of 6 mice receiving PBS alone survived infection (**Fig. 6A**). The ability of the unmodified mAbs to enhance DENV in mice was not affected by differences in the interaction between the human Fcγ constant region and the murine FcγR, since murine/human chimeric mAbs in which the human hinge, CH2 and CH3 domains were replaced with the homologous, murine Igγ2a constant region enhanced the DENV-infection comparably *in vitro* and *in vivo* (data not shown).

To determine whether survival associated with transfer of the DV82.11 and DV87.1 LALA variants was associated with reduced viral load, we measured the viral burden in serum and tissues 3.5 days following D2S10 antibody-enhanced infection. Serum viremia and tissue viral load measured in liver, small intestine and lymph node were significantly decreased in mice receiving 5 μg of either LALA variant as compared to mice receiving 5 μg of the parent unmodified mAbs (**Fig. S2**).

To explore whether the LALA variants could serve as a possible therapy following DENV infection, we administered 50 μg of the LALA variants or unmodified mAbs to mice 24 hours after infection with DENV2 D2S10 under enhancing conditions (24 hours after transfer of heterotypic anti-DENV1 serum) (40). Mice receiving either the DV82.11 or DV87.1 LALA variant survived the normally lethal infection, whereas mice receiving the unmodified parent mAbs succumbed to infection, as did mice receiving non-binding isotype control (DV22.3 LALA mAb that only recognizes DENV4) or PBS (**Fig. 6B**). In summary, these results demonstrate that engineering of the LALA mutation on strongly neutralizing mAbs abrogates the capacity for ADE and confers a protective phenotype as a post-exposure therapy in mice.

Discussion

In this study we have shown that individuals that recover from primary or secondary DENV infection have a large repertoire of memory B cells which is skewed towards broadly cross-reactive antibodies specific for DI/II of the E protein while only a minority of memory B cells produce potently neutralizing antibodies that bind to DIII. Out of the fifty mAbs characterized, three were selected based on neutralizing potency and breadth and expressed as recombinant mAbs carrying mutations in the CH2 domain that prevent binding to the FcγRs. These mAbs did nor cause ADE *in vitro* and *in vivo* and showed therapeutic efficacy in a mouse model of antibody-enhanced lethal DENV infection.

This study represents the first attempt to interrogate the human memory B cell repertoire of Dengue immune donors in order to identify the antigenic specificity and cross-reactivity of the antibody response. In the two donors that were analyzed systematically using a broad screening strategy, the memory B cell repertoire appears to be strongly skewed towards recognition of E-DI/II, with relatively fewer B cells specific for E-DIII, pr-M and NS proteins. Our findings that the human memory B cells are primarily skewed for recognition of E-DI/II are consistent showing with serological studies with WNV and DENV that suggest that the human humoral response is directed away from E-DIII (41-44). Moreover, two recent studies described recombinant anti-WNV antibodies that were generated after phage display screening of scFv (V_H-V_L) molecules (45, 46), both of which observed immunodominance of DI/DII mAbs against WNV E protein. It should be noted that binding efficiency and neutralization of DI/II mAbs was higher with the homologous serotype and 2 to10-fold lower with heterologous serotypes, a finding that may explain their role in enhancing heterologous infections. For

uncertain reasons, it appears that E-DIII-specific antibodies are more rare in the human repertoire although they still could contribute significantly to protection (43).

Previous studies using mouse mAbs established that E-DIII is the primary target of the most potent neutralizing antibodies against WNV and DENV (18-20, 42, 47, 48). Our findings in human subjects are in line with these observations, as the E-DIII-specific mAbs isolated were indeed the most potently neutralizing, with EC_{50} values in the low ng/ml range. However, within the limitations of the available data, it appears that the human antibodies to DIII have a considerable breadth being able to neutralize two to three serotypes while antibodies produced by mice immunized by recombinant E can only neutralize a few genotypes within a given serotype (21).

ADE was evaluated *in vitro* by measuring the capacity of the mAbs to augment infection of $Fc\gamma RII^+$ human K562 cells, which are poorly susceptible to DENV infection in the absence of antibodies. All neutralizing antibodies mediated ADE at sub-neutralizing concentration while some E-specific mAbs and most prM-specific mAbs mediated ADE but lacked neutralizing activity. A recent study reported the isolation of three human E protein-specific mAbs, two of which showed enhancing but not neutralizing activity (49). It is possible that neutralization and enhancement are linked directly by their relative stoichiometry of binding to the virion (35, 50). Nonetheless, the percentage of infected cells and the range of mAb concentration that promoted ADE varied widely among the antibodies, independent of their neutralizing capacity.

Most of the prM-specific mAbs described in the present study were highly cross-reactive and showed ADE activity over a broad range of concentrations. Previous studies reported that mouse anti-prM mAbs neutralized infection weakly, if at all, (26, 51-53).

Additionally, some prM-specific mAbs passively protected mice from flavivirus *in vivo,* but the mechanism of protection remains unclear (26, 54-56). Anti-prM mAbs also have been reported to mediate ADE infection in cells with FcγR (51) or without FcγR, (25), indicating a possible role for anti-prM mAbs as auto-antibodies mediating antibody-dependent cell cytotoxicity. Our finding of broadly cross reactive prM-specific human mAbs that promote ADE robustly suggest a potential role this class of antibodies in enhancing DENV infection in humans. Accordingly, recent studies in infants have also implicated anti-prM antibodies in the susceptibility of infants to severe dengue (57). Alternatively, anti-prM antibodies could enhance DENV propagation by enhancing the infectivity of immature, "non-infectious" virions, as was recently reported (27).

Recently, a nine amino acid-deletion in the Fc region of a humanized chimpanzee antibody was shown to abrogate antibody-dependent enhancement of DENV infection *in vitro* and in monkeys (58), and a single amino acid mutation (N297Q) in the Fc region of a humanized mouse mAb that eliminated Fc-γR interaction also abolished ADE of DENV infection and lethal disease in a mouse model (40). These data confirm that ADE causes enhanced disease *in vivo* and can be prevented by modifying the antibody to abolish binding to Fc receptors. Here, we produced recombinant variants of three neutralizing mAbs designed as component of a cocktail capable of targeting two distinct epitopes on each of the four DENV serotypes. These LALA variants, carrying two mutations in the CH2 region that prevent binding to Fc-γR, retained neutralizing activity and lacked completely ADE. When combined *in vitro*, we observed an increase in neutralization potency (two- to four-fold) of all four DENV serotypes, consistent with an additive effect. When tested *in mice,* the LALA variants prevented ADE and disease induced by a

different fusion-loop specific mouse mAb (4G2). Further examination revealed a significant reduction in tissue viral load and serum viremia by the LALA variants, indicating that in the absence of FcγR binding, the LALA variants still efficiently neutralize DENV infection and prevent disease. Thus, even though effector functions can enhance protective efficacy of anti-flavivirus antibodies (28, 47, 59, 60), they are not required for highly neutralizing antibodies.

The development of a vaccine against DENV has been problematic in part due to the possible risk of eliciting suboptimal immune responses that might lead to ADE and severe disease after challenge with heterologous virulent strains. In the absence of an effective DENV vaccine, passive therapy with neutralizing antibodies may provide an alternative for the treatment of dengue. A post-exposure prophylaxis approach is currently being pursued for treatment of several viral diseases such as respiratory syncytial virus (RSV), rabies, hepatitis B and WNV (61). Humanized mAbs obtained from mice or primates have been used for this purpose, but fully human mAbs generated during a natural infection represent an attractive alternative with a reduced risk of cross-reactivity with self-antigens and of stimulation of allo-antibody responses. In an attempt to explore the feasibility of a passive serotherapy approach for dengue, we identified three human mAbs (DV87.1, DV22.3 and DV82.11) that neutralize all four DENV serotypes by targeting two distinct epitopes on each virus. We showed that when tested as a post-exposure therapeutic 24 hours after infection, both the DV82.11 and DV87.1 LALA mAbs protected against an antibody-enhanced lethal infection. Thus, at least in mice, even if ADE is initiated, there is a time window when neutralizing antibodies that lack the ability to enhance infection can still protect against lethal disease. It should be

noted that the LALA substitutions do not affect interactions with the neonatal FcRn (38), thus preserving long half-live and sustained immune protection.

Overall, our results indicate that strongly neutralizing DIII-specific antibodies are relatively rare in the human natural anti-DENV antibody response. Instead, is the response is dominated by cross-reactive, weakly and non-neutralizing antibodies that recognize DI and DII and, in the context of DENV infection, have a high potential for promoting ADE. However, since the vast majority (>99%) of humans experiencing secondary DENV infection do not develop severe life-threatening disease, the E-DI/II-specific antibodies or the rare highly neutralizing E-DIII-specific antibodies are likely protective, along with other components of the innate and adaptive immune system. These results are a foundation for further investigation to address whether antibody-based therapeutic can control severe DENV disease in humans.

Materials and Methods

Reagents. Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Vero and K562 cells were cultured in DMEM or RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Hyclone), 1% penicillin, 1% streptomycin and L-glutamine (all from Invitrogen) (complete DMEM or RPMI). C6/36 cells (*Aedes albopticus* mosquito cells) were cultured at 28°C in 5% CO_2 in EMEM (Invitrogen) and supplements as before. Attenuated DENV vaccine strains used in this work were: rDEN1Δ30 (62), rDEN2/4Δ30 (ME) (63), rDEN3/4Δ30 (ME) (64) and rDEN4Δ30 (65). The DENV2 strain D2S10 used for *in vivo* experiments was derived from the parental DV2 PL046 Taiwanese isolate as described (66). The for mouse 4G2 hybridoma was purchased from ATCC, grown in serum-free medium (Invitrogen), and purified using protein G affinity chromatograpy (Thermo Scientific). To generate mouse anti-DENV sera, six- to eight-week old AG129 mice were infected intra-peritoneally (i.p.) with 10^5 of DENV1 strain 98J, a strain isolated in the Harris laboratory from a patient from Guyana in 1998. Mice were sacrificed by terminal bleed 6-8 weeks after infection, sera collected and stored.

Memory B-cell immortalization and cloning. Peripheral blood samples were obtained from four adult donors who had been diagnosed with DENV infection. The study protocol was approved by the Scientific and Ethical Committee of the Hospital for Tropical Diseases as well as the Oxford Tropical Research Ethical Committee. All patients provided written informed consent. The determination of primary or secondary dengue was made on the basis of acute serology and results of $PRNT_{50}$ assays in late

convalescence. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation and cryopreserved. On the day of use, PBMC were thawed and B cells were enriched using CD22 microbeads (Miltenyi) and IgG^+ memory B cells were further purified by cell sorting using a FACSAria (BD Biosciences) as described (32). Cells were immortalized using EBV and CpG oligodeoxynucleotide 2006 (Microsynth) as described and cultured at 20 cells/well in multiple 96-well plates with irradiated allogeneic PBMC in complete RPMI. After 2 weeks the culture supernatants were screened for the presence of DENV-specific Abs. Positive cultures were expanded and cloned by limiting dilution.

Intracellular staining with DENV-infected cells. C6/36 cells or Vero cells were infected with the DENV3 isolate recovered from Donor 76, with the DENV1 isolate recovered from Donor 12, or with attenuated DENV1, 2, 3, and 4 viruses at MOI leading to 70-90% of infected cells. After 5 days, cells were harvested, fixed with 2% formaldehyde (Sigma), and permeabilized in PBS containing 1% FCS and 0.5% saponin (Sigma). Fixed and permeabilized DENV-infected cells were incubated for 1h on ice with B cell culture supernatants, washed and stained with Cy5-conjugated goat anti-human IgG (diluted 1:500) (Jackson Immunoresearch). Cells were analyzed on a FACSArray or FACSCanto equipped with high throughput sampler (BD Biosciences).

ELISA. 96-well micro-ELISA plates (Corning) were coated overnight at 4°C with recombinant E protein from DENV1-4 (67) or with recombinant E-DIII from the DENV3 of Donor 76 (68) (all at 1µg/ml in 0.1M Na carbonate buffer, pH 9.3). After washing and

blocking, serial dilutions of mAbs in 10% FCS-PBS and added for 1h at 37°C. After further wash, mAbs bound were revealed using goat anti-human IgG coupled to alkaline phosphatase (diluted 1:500) (Jackson Immunoresearch). In same experiments ELISA plates were coated with the mouse mAb 4G2 (1 µg/ml overnight) followed by a mixture of DENV1-4 antigens. ELISA with recombinant non-structural protein were performed as described (69).

Yeast expression and staining. Yeast expressing E-DI/II and E-DIII of DENV2 were generated as previously described (18, 22). Briefly, yeast expressing the amino acid sequence of E-DI/II (residues 1 to 295) and E-DIII (residues 296 to 415) of DENV2 (strain 16681) were made by engineering BamHI and XhoI restriction enzyme sites at the 5' and 3' ends of the ectodomain construct using PCR amplification. This fragment was cloned into the BamHI and XhoI sites of the pYD1 vector (Invitrogen) and expressed in the *S. cerevisiae* strain EBY100. Yeast cells expressing wild type or mutant E-DI/II or E-DIII of DENV or WNV (strain New York 1999) were stained with 50 µl of human mAb supernatant on ice for 30 min. The cells were then incubated with a 1:500 dilution of goat anti-human IgG conjugated to Cy5 (Jackson Immunoresearch) for 30 min on ice. After fixation with 1% formaldehyde (Sigma) in PBS, yeast cells were analyzed by flow cytometry.

Immunoblot analysis. Equal amounts of concentrated supernatant from DENV3- or DENV2-infected or uninfected Vero cells were separated under non-reducing conditions by SDS-PAGE and transferred onto nitrocellulose membranes. Filters were blocked with

10% dry skim milk and probed with monoclonal antibodies at 37°C. Blots were incubated with horseradish peroxidase–conjugated mouse anti-human IgG or donkey anti-rabbit IgG secondary antibody and developed with ECL Plus Western Blotting Detection System (GE Healthcare). E protein was identified using DV55.1, NS1 protein using a rabbit anti-NS1 serum. The protein prM and C were identified as 20-kD and 10-15 kd bands, respectively.

Production of recombinant IgG wild-type and LALA mutants. RNA was isolated with RNeasy kit (Qiagen) from EBV-immortalized B cell clones DV82.11, DV87.1, and DV22.3 and cDNA synthesized with M-MLV reverse transcriptase (Invitrogen). Variable regions of heavy-chain and light-chain genes were sequenced and cloned by PCR into human Igγ1, Igκ and Igλ expression vectors (provided by M. Nussenzweig, Howard Hughes Medical Institute, The Rockefeller University, New York) using gene-specific primers and Pfu Turbo (Stratagene) (70). The vectors contain a murine Ig gene signal peptide sequence and a multiple cloning site upstream of human Igγ1, Igκ and Igλ constant regions, with either wild-type or with leucine-to-alanine mutations at positions CH2 1.3 and 1.2 of Igγ1, according to the IMGT unique numbering for C-DOMAIN (LALA mutant) (38) introduced by site-directed mutagenèsis (GenScript). Equal amounts of heavy and light chain vectors were mixed with an equal amount of polyethylenimine (Sigma), incubated for 15 min and added to 80% confluent HEK293T cells (ATCC) in DMEM supplemented with 1% Nutridoma (Roche). Antibodies were recovered from supernatants harvested 3 days after transfection and purified using protein A affinity chromatography followed by sephadex 200 size exclusion chromatography.

Infection neutralization and enhancement. DENV neutralization was measured using a flow based assay. The day before the infection, 5,000 Vero cells were plated in 96-well flat-bottom micro-plates. Different dilutions of mAb were mixed with DENV (all used at an MOI of 0.04) in a final volume of 60µl for 1h at 37 °C and then added to Vero cells monolayers. After three days the cells were fixed with 1% formaldehyde, permeabilized in PBS 1% FCS 0.5% saponin, and stained with mouse mAb 4G2 (71) directed against the fusion loop of all DENV E proteins (23). The cells were incubated with a 1:200 dilution of goat anti-mouse IgG conjugated to R-phycoerythrin (SouthernBiotech) and analyzed by flow cytometry. ADE was measured by a flow assay using K562 cells, a human erythroleukemia line that expresses FcγRIIA and is resistant to DENV infection in the absence of enhancing antibodies. Monoclonal antibodies and DENV (at an MOI of 0.04) were mixed in a final volume of 60µl for 1 hour at 37 °C and added to K562 cells (5000 cells/well). After three days at 37°C, cells were fixed, permeabilized, and stained with mouse mAb 4G2 as above and the number of infected cells was determined by flow cytometry as above. EC_{50} values were determined by nonlinear regression analysis using a variable slope (GraphPad Prism 5).

Determination of neutralization synergy. The increase in neutralization achieved by combinations of neutralizing mAbs was assessed using a variable antibody ratio approach (39). One antibody was serially diluted and then a fixed amount of a second neutralizing antibody at a weakly neutralizing concentration was added (to standardize the amount of that antibody bound to the virus). Attenuated DENV (at an MOI of 0.04) was mixed with

antibodies and added to Vero cells after an incubation period of 1h at 37 °C. Three days after infection, cells were analyzed by flow cytometry. The change in neutralization potency was assessed by comparing the EC_{90} obtained with a single antibody titration performed during the same assay.

Effect of LALA antibodies on ADE. Potent enhancing antibodies (0.1μg/ml) or serum from primary dengue-infected donors were mixed with an equal volume of LALA cocktail (DV82.11 =2μg/ml; DV87.1=DV22.3= 0.2μg/ml). Attenuated DENV 1-4 virus strains (at MOI of 0.04) and the antibody were mixed, added to K562 cells, and incubated for three days. Cells were stained with mouse 4G2 and analyzed by flow cytometry as describe above.

Infection of AG129 mice. 129/Sv mice lacking the interferon α/β and γ receptors (AG129) (72) were bred in the UC Berkeley Animal Facility. All experimental procedures were pre-approved by the UC Berkeley Animal Care and Use Committee (ACUC) and were performed according to the guidelines of the UC Berkeley ACUC. Mice were injected i.p. with mAb, PBS, or anti-DENV sera in a total volume of 200 μl, then infected 18-24 hours later with either 10^5 or 10^6 pfu of DENV strain D2S10 diluted in 100μl volume by intravenous (i.v.) injection into the tail vein. In some experiments, 50μg of mAb were transferred to mice i.v. 24 hours post-infection to assess the therapeutic efficacy of the mAb.

Quantitation of virus in tissues and serum. Viral load was determined in the indicated tissues as previously described (73), and expressed as pfu/g of solid tissues. Viral copy number was quantified by qRT-PCR in serum and liver samples. Viral RNA was extracted from 60µl of serum using the QIA-Amp RNA recovery kit (Qiagen). Liver samples were removed and saved in RNA-Later (Ambion) at 4°C prior to processing. Total RNA was extracted using the RNEasy kit (Qiagen) according to the manufacturer's instructions. Quantitation of viral RNA was performed using Taqman reagents (One Step RT-PCR Kit, Applied Biosystems, Foster City, CA) and an ABI PRISM 7700 sequence detection system as previously described (74). Standardization of cellular RNA levels from liver samples was performed using a Taqman rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control kit (Applied Biosystems) and compared to a standard curve based on known amounts of total mouse RNA provided by the manufacturer (73). Serum viremia levels were expressed as pfu equivalents/ mL as described previously (73), and liver viral load as pfu equivalens/µg total mouse RNA.

Statistical analysis. All data were analyzed with Prism software (GraphPad Software). Kaplan-Meier survival curves were used to display mortality data, and log rank analyses were used to determine statistical significance between experimental groups.

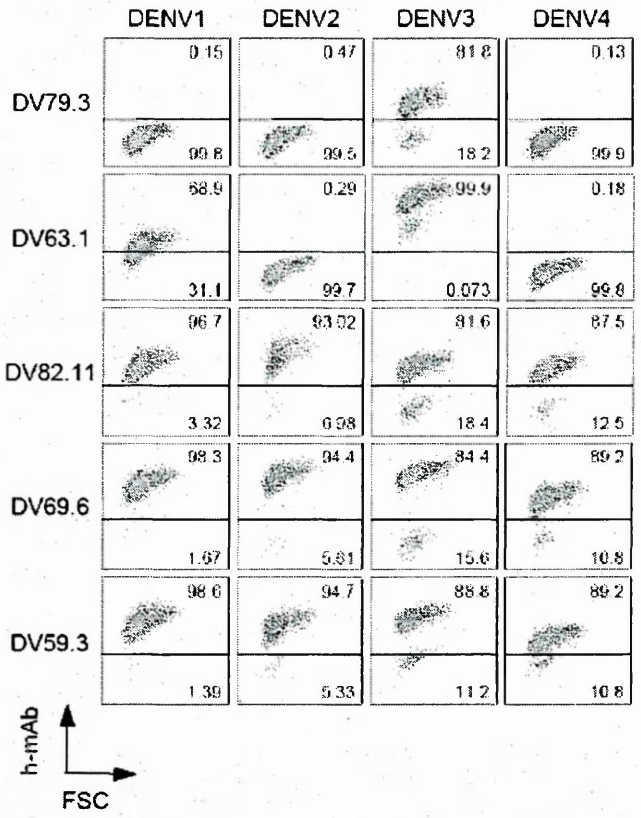

Figure 1. Staining of infected and permeabilized Vero cells identify antibodies to dengue virus. Vero cells were infected by DENV1, 2, 3, and 4 vaccine strains, fixed, permeabilized, and stained with EBV-transformed B cell culture supernatants, followed by fluorescently-labelled anti-human IgG polyclonal antibodies. Representative dot plots show staining by mAbs isolated from Donor 76 reactive against DENV3 alone (DV79.3), DENV1 and DENV3 (DV63.1) or cross-reactive against the four DENV serotypes. Numbers in quadrants indicate the percentage of positive cells. The data shown are from one of two independent experiments performed in with similar results

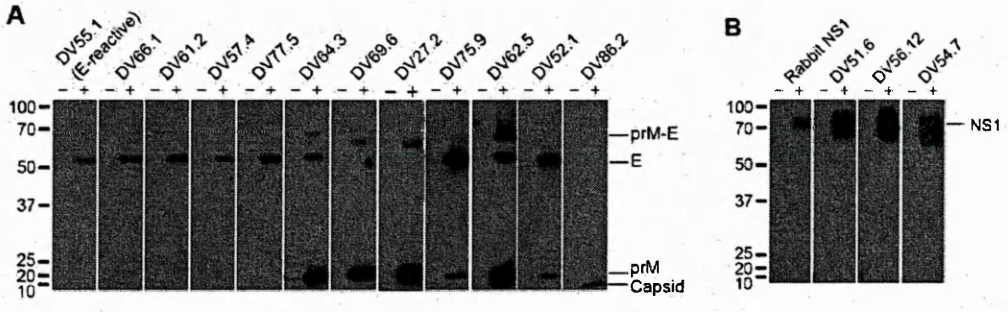

Figure 2. Characterization of antigen specificity by Western blot. mAbs that were not reactive by ELISA were subjected to Western blot analysis. Concentrated supernatants of uninfected control (—) or DENV3-infected Vero cells (+) were separated under non-reducing conditions. E protein was identified by DV55.1, specific for E protein in ELISA (**A**). NS1 protein was identified by a rabbit anti-NS1 serum (**B**). prM and capsid migrate with apparent molecular weights of 20 and 16 kD, respectively. The data shown are from one of at least two independent experiments with similar results.

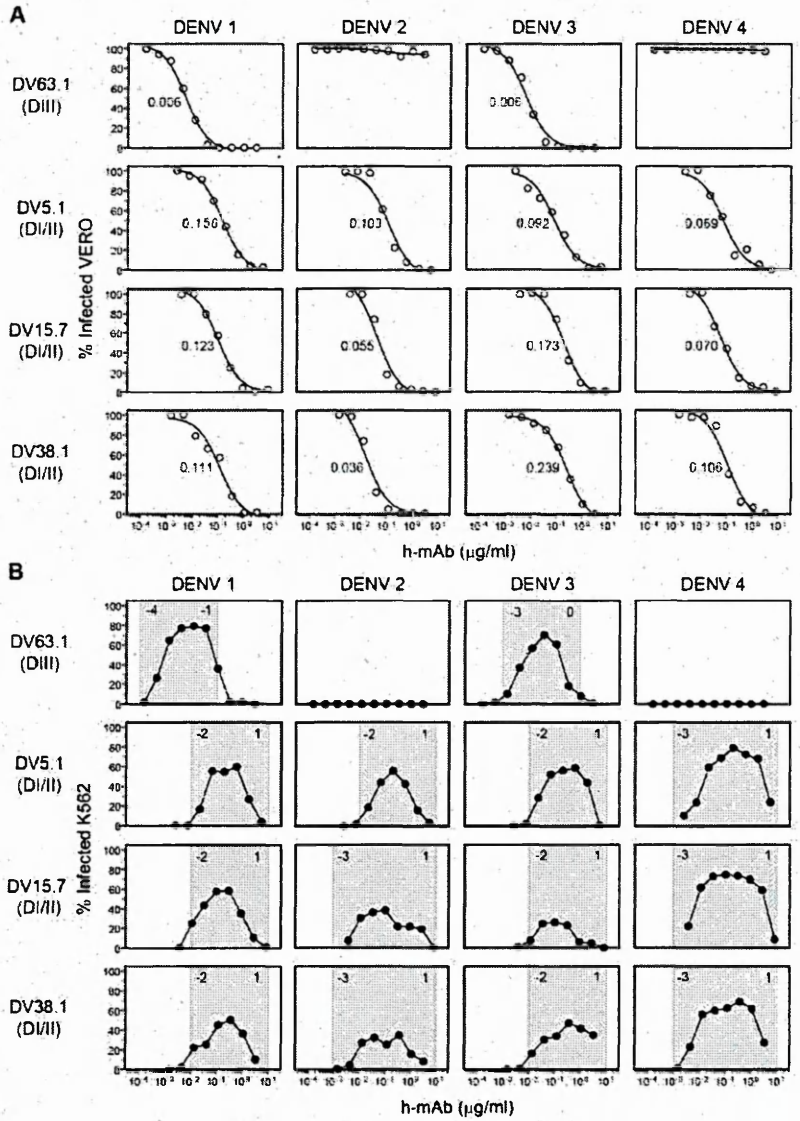

Figure 3. Efficiency of neutralization and enhancement by E-DIII and E-DI/II-specific mAbs. A) For viral neutralization, serial dilutions of mAbs were incubated with DENV1-4 before addition to Vero cells. Shown is the percentage of infected cells after 3 days as a function of increasing mAb concentration (empty circles). Nonlinear regression analysis was used to calculate the EC_{50}, indicated in the panel. **B)** For infection enhancement, serial mAb dilutions were incubated with DENV1-4 before addition to K562 cells. Shown is the percentage of infected K562 cells (filled circles). The range of mAb concentrations where infection enhancement was observed is indicated in gray. Numbers in the gray areas represent logarithm power. The data shown are from one representative experiment out of three independent experiments performed.

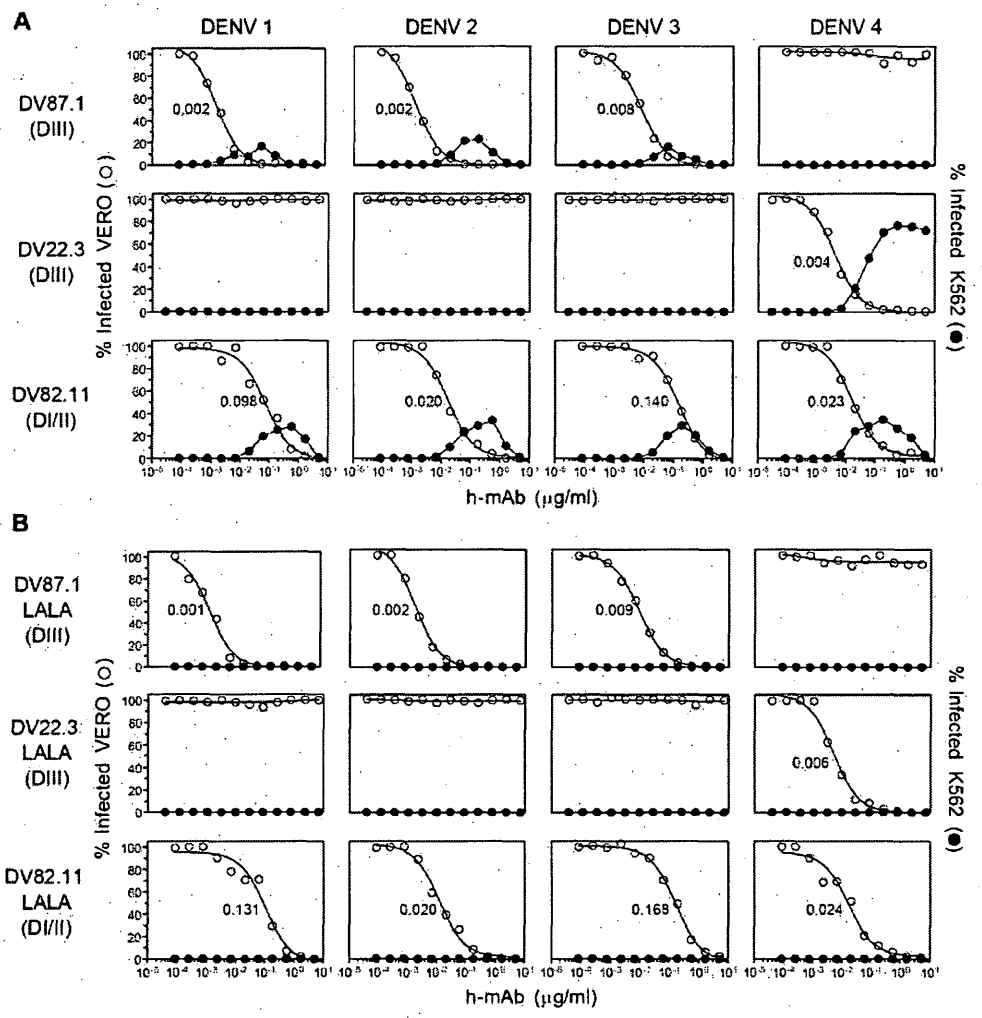

Figure 4. Three mAbs neutralize all four DENV serotypes by binding two distinct epitopes on each virus. Three mAbs (all IgG1) were selected for their specificity and neutralizing activity: DV87.1 binds E-DIII of DENV1, 2 and 3; DV22.3 binds DENV4, and DV82.11 cross-reacts with E-DI/II of all DENV serotypes. **A)** Neutralization (empty circles) and enhancement (filled circles) of infection by recombinant wild-type mAbs. **B)** Neutralization (empty circles) and enhancement (filled circles) of infection by engineered LALA variant mAbs. Nonlinear regression analysis was used to calculate the EC_{50}, indicated in the panel. Data are mean of duplicates. One representative experiment out of three performed.

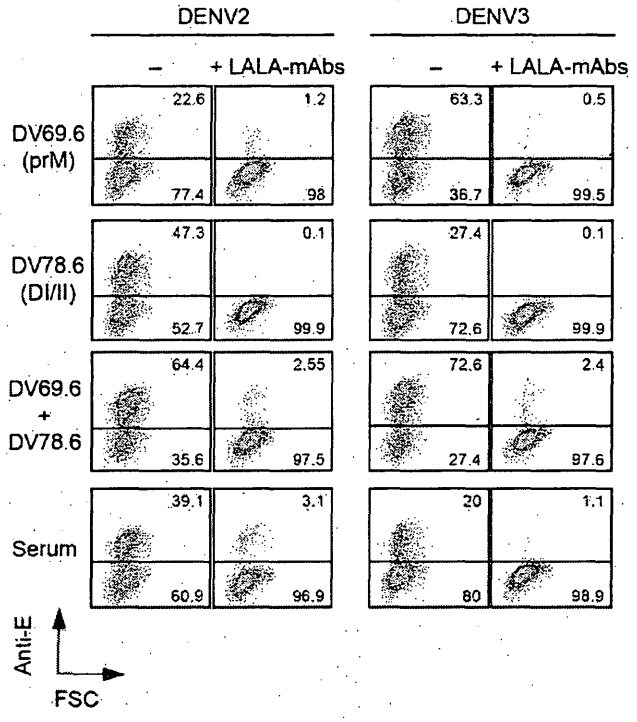

Figure 5. LALA cocktail reverses the effect of enhancing antibodies. LALA cocktail (DV82.11= 2μg/ml, DV87.1=DV22.3= 0.2μg/ml) was used in combination with mAbs directed against prM or DI/II E protein or serum from primary DENV-infected donors at potent enhancing concentration (0.1μg/ml) on K562 cells. The data shown are from one experiment representative of two performed.

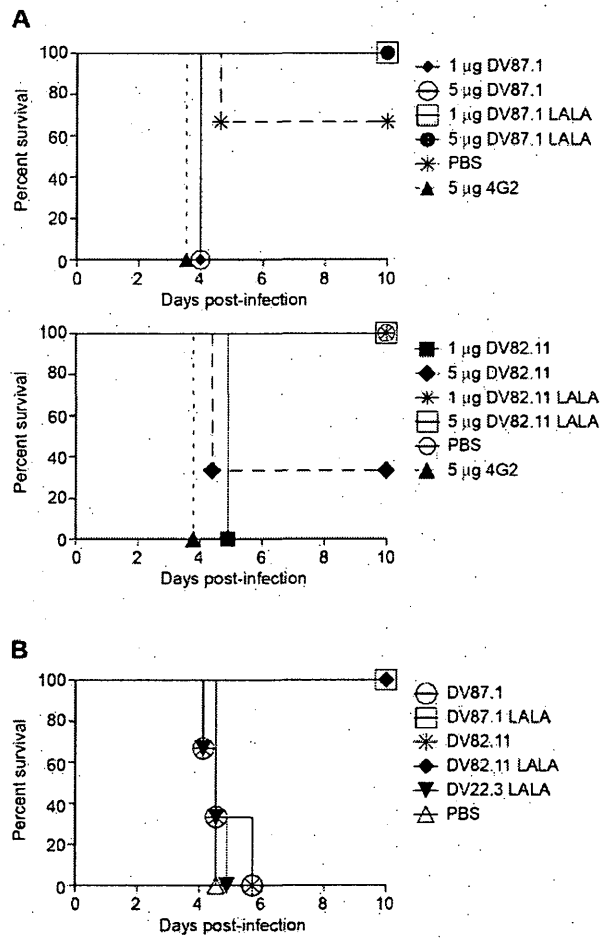

Figure 6. LALA variants do not enhance DENV *in vivo*. A) Upper panel. 1 or 5 µg of DV82.11, DV82.11 LALA variant, mAb 4G2 or PBS were transferred i.p. in 200µl volume into AG129 mice (n=3/group). The mice were subsequently infected 18 to 24 h later with 10^6 pfu DENV2 D2S10. Mortality was monitored for ten days. **Lower panel.** 1 or 5 µg of DV87.1, DV87.1 LALA variant, mAb 4G2 or PBS were transferred i.p. in 200µl volume into AG129 mice (n = 3/group). The mice were infected 18-24 h later with 10^6 pfu DENV2 strain D2S10, and mortality was monitored daily for ten days. **B)** AG129 mice (n=3/group) were administered 35 µl anti-DENV1 98J serum i.p. and were infected 24 hours later with 10^5 DENV2 D2S10 i.v.. Twenty-four hours after infection, the mice were treated with 50 µg of either DV87.1, DV87.1 LALA, DV82.11, DV82.11 LALA, DV22.3 LALA or PBS i.p, and mortality was assessed daily for ten days.

Donor	Infecting serotype	Days p.i.	B cell immortaliz. efficiency	Primary screening	Positive culture/total (%)	DENV-specific B cells
Don 76	Primary DENV3	241	14%	Staining of infected C6/36 cells	434/960 (45%)	16%
Don 7	Primary DENV4	200	9.1%	ELISA E4 protein	81/2844 (2.8%)	1.5%
Don 12	Secondary DENV1	510	13.8%	Staining of infected C6/36 cells	245/1632 (15.6%)	5.4%
Don 92	Secondary DENV2	212	9%	ELISA DENV1-4 lysates	344/2208 (15.6%)	8.6%

Table 1. DENV immune donors and repertoire analysis. PBMC were obtained from donors after a primary or secondary DENV infection and frozen until the day of use. The infecting serotype and the days post infection are reported. IgG^+ memory B cells were immortalized, and cultures secreting DENV-specific mAbs were identified using primary screening methods. The efficiency of immortalization of IgG^+ B cells was estimated and the fraction of positive culture are indicated. The frequency of DENV specific B cells are reported.

mAbs	Donor	Isotype	Specificity	ELISA E protein					Yeasts DV2		Neutralization (EC_{50} µg/ml)				Range of enhancement (log10 µg/ml)			
				DV 1	DV 2	DV 3	DV 4	DIII DV 3	DI/ DII	DIII	DV 1	DV 2	DV 3	DV 4	DV 1	DV 2	DV 3	DV 4
DV 1.1	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.209	0.066	0.262	0.183	-2,1	-2,1	-2,1	-2,1
DV 4.4	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.248	0.214	0.167	0.760	-2,1	-2,1	-1,1	-2,1
DV 5.1	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.156	0.103	0.092	0.069	-2,1	-2,1	-2,1	-3,1
DV 6.1	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.382	0.296	0.351	0.170	-1,1	-2,1	-1,1	-2,1
DV 7.5	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.084	0.075	0.130	0.031	-2,1	-2,1	-2,1	-2,1
DV 8.1	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.103	0.022	0.031	0.024	-3,2	-2,1	-3,1	-3,0
DV 13.4	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.063	0.023	0.016	0.045	-2,1	-3,0	-3,0	-3,0
DV 14.5	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.053	0.049	0.060	0.040	-2,0	-2,0	-2,0	-3,0
DV 15.7	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.123	0.055	0.173	0.070	-2,1	-3,1	-2,1	-3,1
DV 16.5	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.248	0.089	0.219	0.101	-2,1	-2,1	-2,1	-2,1
DV 17.6	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.218	0.103	0.256	0.087	-2,1	-2,1	-2,1	-2,2
DV 18.4	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.670	0.241	0.799	0.699	-2,1	-2,1	-2,1	-2,1
DV 19.3	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.631	0.189	0.368	0.114	-2,1	-2,1	-2,1	-2,1
DV 20.1	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.244	0.128	0.777	0.226	-2,1	-2,1	-1,1	-2,1
DV 21.1	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.161	0.055	0.069	0.101	-2,1	-2,1	-3,1	-3,1
DV 28.8	Don 92	γ1, κ	E, DI/DII	+	÷	+	÷	—	÷	—	0.106	0.048	0.233	0.092	-2,0	-2,0	-2,0	-2,1
DV 38.1	Don 92	γ1, λ	E, DI/DII	+	÷	+	÷	—	÷	—	0.111	0.036	0.239	0.106	-2,1	-3,1	-2,1	-3,1
DV 78.6	Don 76	γ1, κ	E, DI/DII	+	÷	+	÷	—	n.d.	—	0.591	0.251	0.809	0.367	-2,1	-2,1	-2,1	-2,1
DV 82.11	Don 76	γ1, λ	E, DI/DII	+	÷	+	÷	—	n.d.	—	0.043	0.024	0.090	0.117	-2,1	-2,1	-2,1	-2,1
DV 90.3	Don 76	γ1, λ	E, DI/DII	+	—	+	—	—	n.d.	—	0.342	—	0.454	—	-2,1	—	-1,1	—
DV 74.4	Don 76	γ1, λ	E, DI/DII	—	—	+	—	—	n.d.	—	—	—	0.020	—	—	—	-2,0	—
DV 79.3	Don 76	γ1, κ	E, DI/DII	—	—	+	—	—	n.d.	—	—	—	0.023	—	—	—	-2,0	—
DV 76.5	Don 76	γ1, κ	E, DI/DII	—	—	+	—	—	n.d.	—	—	—	—	—	-1,2	-2,1	-2,1	-2,1
DV 55.1	Don 76	γ1, λ	E, DIII	+	+	+	—	+	n.d.	+	0.013	0.577	0.014	—	-3,0	-1,1	-3,1	—
DV 87.1	Don 12	γ1, κ	E, DIII	+	+	+	—	+	n.d.	+	0.004	0.004	0.008	—	-3,1	-2,1	-2,0	—
DV 63.1	Don 76	γ1, κ	E, DIII	+	—	+	—	+	n.d.	—	0.006	—	0.006	—	-4,-1	—	-3,0	—
DV 22.3	Don 7	γ1, λ	E	—	—	—	+	—	n.d.	—	—	—	—	0.006	—	—	—	-2,1
DV 66.1	Don 76	γ1, κ	E	—	—	—	—	—	n.d.	—	—	—	1.950	—	—	—	-2,1	—
DV 61.2	Don 76	γ1, κ	E	—	—	—	—	—	n.d.	—	—	—	—	—	0,2	-2,1	-2,1	-2,1
DV 57.4	Don 76	γ1, κ	E	—	—	—	—	—	n.d.	—	—	0.200	0.167	0.193	—	-3,0	-3,0	-2,0
DV 77.5	Don 76	γ1, κ	E	—	—	—	—	—	n.d.	—	—	—	—	—	-1,2	-2,0	-2,1	-2,1

Table 2. Characterization of E-reactive human mAbs. The table reports the donor from which the mAbs were isolated and the mAb isotype and light chain usage as determined by specific ELISA. The target specificity of the mAbs was determined by ELISA performed with recombinant E proteins (from DENV1-4 serotypes). Four mAbs did not recognize E proteins by ELISA but bound E protein in Western blot. Viral neutralization was performed using Vero cells and attenuated DENV1-4 viruses. Shown are EC_{50} values. Enhancement of infectivity was performed using K562 cells and attenuated DENV1-4 viruses. Shown is the range of mAb concentrations where infection enhancement was observed (see example in Fig. 3). The data shown are representative of two independent experiments.

mAbs	WNV					
	E	DIII	DI/II	W101R	G106E	W233F
DV 5.1	+++	–	+++	–	–	+++
DV 6.1	–	–	++	+	+	–
DV 14.5	+++	–	+++	–	–	+++
DV 15.7	+++	–	+++	++	+++	+++
DV 16.5	+++	–	+++	+++	+++	+++
DV 18.4	–	–	+++	–	–	+++
DV 19.3	+	–	+++	+	+++	+++

Table 3. Cross-reactive E mAbs bind DI/II and map to fusion loop. Some cross-reactive E specific mAbs stained yeast expressing DI/II of WNV. Mutations in conserved residues (W101R, G106E) abolished the ability of three mAbs to bind to E protein. DV6.1 showed decreased binding with E containing the W233F mutation.

mAbs	Donor	Isotype	Specificity	Neutralization (EC_{50} μg/ml)				Range of enhancement (log10 μg/ml)			
				DV 1	DV 2	DV 3	DV 4	DV 1	DV 2	DV 3	DV 4
DV 64.3	Don 76	γ1, κ	prM	0.327	0.402	0.067	0.128	-4 , 1	-4 , 1	-4 , 1	-3 , 1
DV 69.6	Don 76	γ1, λ	prM	0.912	1.559	0.129	0.070	-4 , 1	-3 , 1	-4 , 1	-4, 2
DV 27.2	Don 92	γ1, κ	prM	0.391	0.345	0.110	0.076	-3 , 1	-3 , 1	-3 , 1	-3 , 1
DV 75.9	Don 76	γ1, κ	prM	–	–	–	–	-2 , 0	-1 , 2	-3 , 0	-2 , 0
DV 62.5	Don 76	γ1, λ	prM	–	–	–	–	-2 , 1	-2 , 1	-2 , 1	-2 , 1
DV 52.1	Don 76	γ1, κ	prM	–	–	–	–	–	–	-3 , 1	–
DV 86.2	Don 76	γ1, λ	capsid	–	–	–	–	–	–	–	–
DV 34.4	Don 92	γ1, κ	n.d.	–	0.010	–	0.431	–	-3 , 0	–	-1 , 2

Table 4. Characterization of non-E-reactive mAbs. Out of fifty mAbs isolated, seven mAbs 92 bound prM protein and one bound the C protein as determined by Western blot assay (See Fig. 2). The specificity of mAb DV34.4 was not assigned. The mAb isotype and light chain usage were determined as described in Table 2. Viral neutralization and enhancement assays were performed on Vero and K562 cells, respectively, using attenuated DENV1-4 viruses. Shown are EC_{50} values of neutralization and range of mAb concentrations of infection enhancement. The data shown are representative of two independent experiments.

mAbs	Donor	Isotype	ELISA	
			NS1	NS3
DV 70.1	Don 76	γ1, κ	+	–
DV 54.7	Don 76	γ1, κ	+	–
DV 60.3	Don 76	γ1, κ	+	–
DV 56.12	Don 76	γ1, λ	+	–
DV 59.3	Don 76	γ4, λ	+	–
DV 53.4	Don 76	γ1, λ	+	–
DV 51.6	Don 76	γ1, λ	+	–
DV 65.5	Don 76	γ1, κ	–	+
DV 67.9	Don 76	γ1, λ	–	+
DV 71.1	Don 76	γ1, λ	–	+
DV 68.2	Don 76	γ3, λ	–	+

Table 5. Reactivity of human mAbs against DENV non-structural proteins. Out of fifty mAbs isolated, 11 bound NS proteins. The mAb isotype and light chain usage were determined by ELISA. Antigenic specificity was determined by ELISA using recombinant NS1, NS3, NS4B and NS5 proteins.

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Appendix 2. Models constructed from clinical parameters at enrolment

Results from Bayesian model averaging using clinical parameters at enrolment (Figure 7-1) indicate that additional covariates that are included in models with reasonable plausibility are sex, vomit, tenderness, and liver enlargment. The best model is model 1 including age and present of bleeding sign.

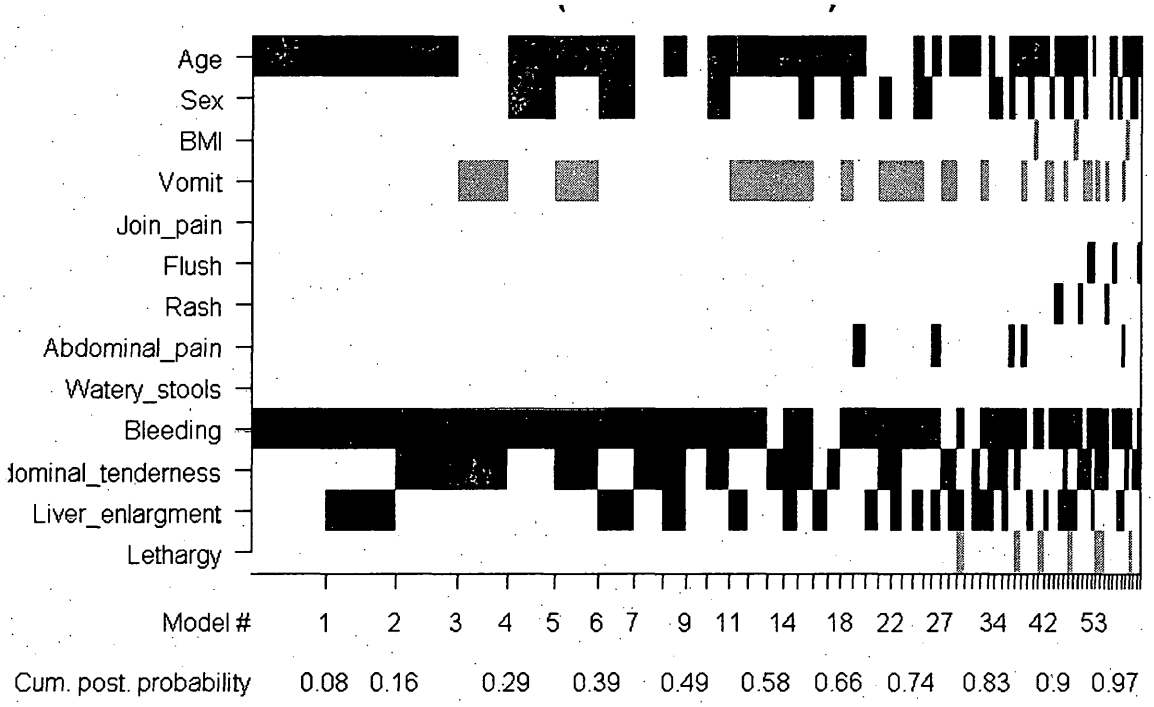

Figure 7-1: Prognostic models constructed from clinical covariates by Bayesian model averaging (BMA).

Black and gray rectangles, respectively, correspond to covariates included in the model with a positive or negative association with outcome while blank spaces mean covariates that aren't included in the model. Cum.post.probability represents as cumulate posterior probability. The posterior probability here means the probability of the model that is assigned after the relevant covariate is taken into account.

Appendix 3. Models constructed from haematological parameters at enrolment

Results from Bayesian model averaging are displayed in Figure 7-2. According to this figure, the selected model (model 1) has a posterior probability of 0.15 but other models which exclude hematocrit (model 2, posterior probability 0.13), or exclude hematocrit and add lymphocyte count (model 3, posterior probability 0.09) would also be plausible given the data. The best model is model 1 including haematocrit, platelet, white cell count, and ALT>40 U/L.

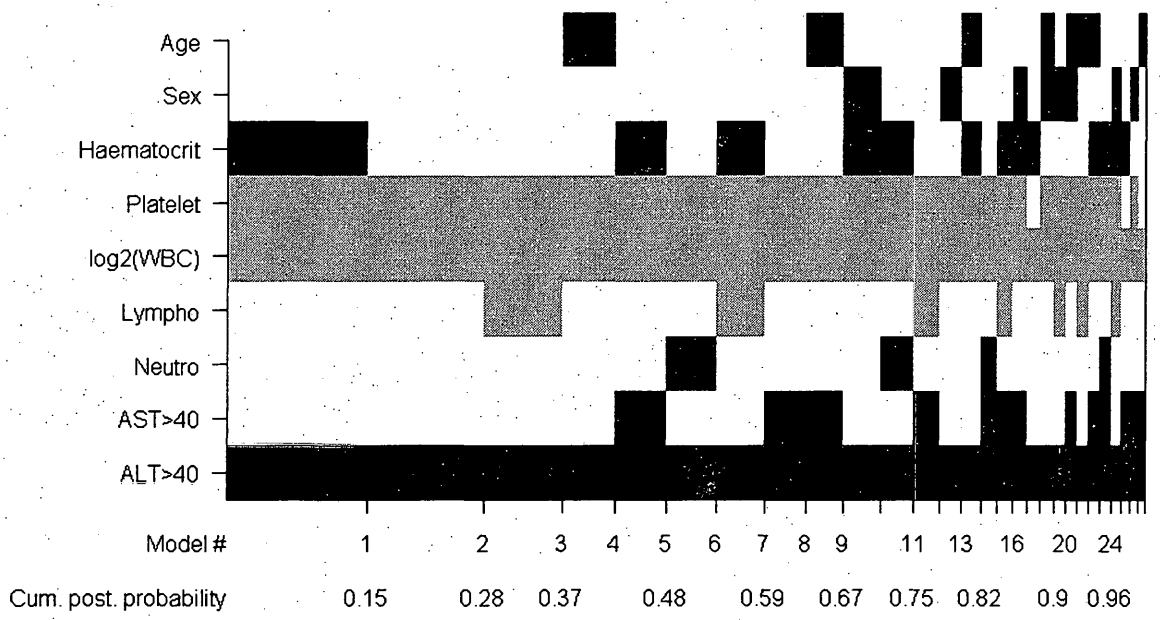

Figure 7-2: Prognostic models constructed from haematological parameters by Bayesian model averaging (BMA).

Black and gray rectangles, respectively, correspond to covariates included in the model with a positive or negative association with outcome while blank spaces mean covariates that aren't included in the model. Cum.post.probability represents as cumulate posterior probability. The posterior probability here means the probability of the model that is assigned after the relevant covariate is taken into account.

Appendix 4. Models constructed from clinical and haematological parameters at enrolment

Results from Bayesian model averaging using clinical and haematological parameters at enrolment altogether (Figure 7-3) show model 1 has the highest posterior probability and is the simplest model.

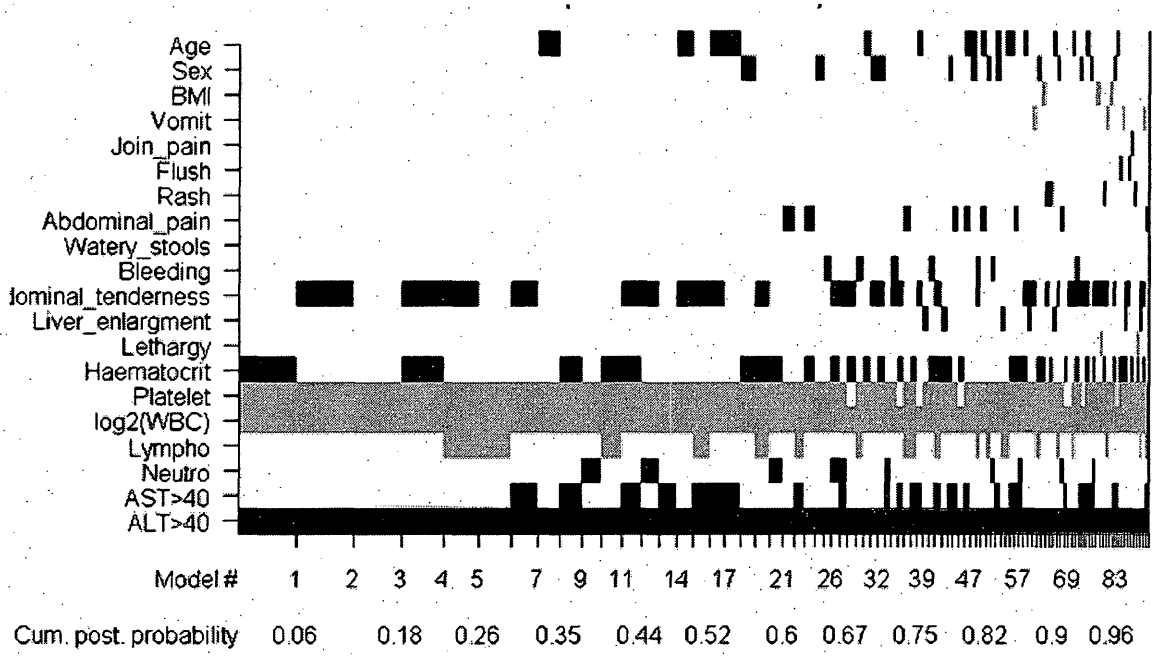

Figure 7-3: Prognostic models constructed from clinical haematological parameters by Bayesian model averaging (BMA).

Black and gray rectangles, respectively, correspond to covariates included in the model with a positive or negative association with outcome while blank spaces mean covariates that aren't included in the model. Cum.post.probability represents as cumulate posterior probability. The posterior probability here means the probability of the model that is assigned after the relevant covariate is taken into account.

Appendix 5. Clasification tree generated using the clinical signs and symptoms at enrolment for predicting DHF

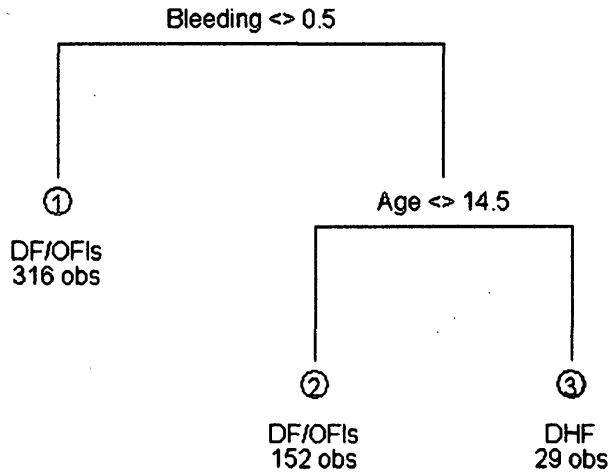

Figure 7-4: Clinical classification tree for predicting DHF at early illness.

Table 7-1: Perfomance of the clinical classification tree

Sesitivity	18/109 (0.17)
Specificity	377/388 (0.97)
PPV	18/29 (0.62)
NPV	377/468 (0.81)
Accuracy	395/497 (0.79)

Appendix 6. Clasification tree generated using the haematological profile at enrolment for predicting DHF

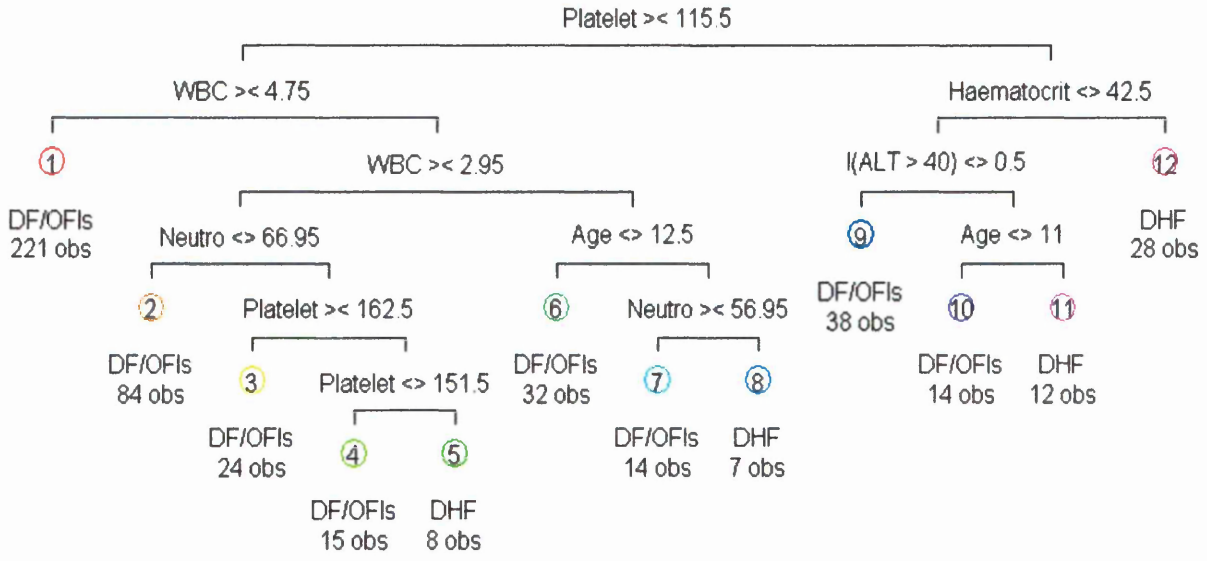

Figure 7-5: Classification tree for predicting DHF based on haematological profile at enrolment.

Table 7-2: Perfomance of the haematological classification tree

Sesitivity	45/109 (0.41)
Specificity	378/388 (0.97)
PPV	45/55 (0.82)
NPV	378/442 (0.86)
Accuracy	423/497 (0.85)

Appendix 7. Case report form of the early clinical and laboratory features study

Study Code FG

CLINNICAL, VIRAL, GENETIC AND IMMUNOLOGICAL RISK FACTORS FOR DSS

Screening page

Inclusion criteria (please check box):

1. Dengue is a possible diagnosis ☐
2. Patient is aged between 2-18 years ☐
3. They have a history of symptoms of <72hrs ☐
4. Their parents give fully informed assent ☐

Enrolling doctor's signature: _____

Date: _____

Demographic Information at Enrolment

Name of patient:_____ Name of patient's mother (father) _____

Address (within 2 weeks before admission to hospital): _____

Name of school _____ Boarding-school [__] Day-boarding school [__] Other [__]

Date of enrolment [__I__I/__I__I/[__I__] *dd/mm/yy* Time of enrolment [__I__I/__I__] hrs/min

Date of Fever onset [__I__I/__I__I/[__I__] *dd/mm/yy* *Approx.* Time of fever onset [__I__I/__I__] hrs/min

Date of birth [__I__]/[__I__]/[__I__] *dd/mm/yy* Sex [__] Male [__] Female

(if date of birth is unknown) Age: [__I__] (YY)

Weight on enrolment [__I__I__]. [__] kg Height [__I__I__] cm

Recent Medical History (chief "complaints" which led to presentation) –

Fever [__] Vomiting [__] Diarrhoea [__] Muscle pain [__] Joint pain [__] Retroorbital pain [__]

Severe abdominal pain [__] Bleeding [__] Other [_____]

Referral and previous management (DK="don't know")

Is the patient referred from another **INPATIENT** health care facility? [__] no [__] yes

Is the patient referred from an **OUTPATIENT** health care facility? [__] no [__] yes

If 'yes', did the patient receive intravenous fluid in the last 24 hours? [__] no [__] yes [__] DK

Has the patient taken aspirin during the week before enrolment? [__] no [__] yes [__] DK

Pregnancy present? [__] no [__] yes [__] DK

Past Medical History (DK="don't know")

Diabetes [__] no [__] yes [__] DK Asthma [__] no [__] yes [__] DK

Other [__] no [__] yes - if 'yes', please specify: [_____]

Status at Discharge (to be filled in at discharge!)

Date of discharge/death: [__I__]/[__I__]/[__I__] *dd/mm/yy*

Outcome: [__] Full recovery without complication. If NO insert a code here [__]

(1 – Incomplete recovery; 2 – Left against medical advice; 3 – Transferred ; 4 – Death
5 – Other (specify): _____)

Clinician's final diagnosis:

This is a case of dengue **only** [__] no [__] yes (If 'no', please continue)

This is a case of dengue plus other diagnosis or complication 1: [__] no [__] yes

If 'yes' please specify diagnosis/complication 1: [_____]

This is **NOT** a case of dengue, but other main diagnosis: [_____]

Clinical Assessment – GENERAL: to be filled in at the end of each 24 hour period (each 24 hour period starts at [__I__]/[__I__] hours) **Lâm sàng – KHÁM TỔNG QUÁT: điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút)**				
Date of assessment (dd/mm/yy) **Ngày khám (ngày/tháng/năm)**	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
DAY (1 is the enrolment day) **Ngày** (ngày 1 là ngày bắt đầu nghiên cứu)	**Ngày [ ]**	**Day [ ]**	**Day [ ]**	**Day [ ]**
Place of assessment *(Nơi khám)*	Ngoại trú [ ] Nội trú [ ]	Outpatient [ ] Inpatient [ ]	Outpatient [ ] Inpatient [ ]	Outpatient [ ] Inpatient [ ]
Eating normally *(Ăn bình thường)*	[ ] Không [ ] Có	[ ] no [ ] yes	[ ] no [ ] yes	[ ] no [ ] yes
Drinking normally *(Uống bình thường)*	[ ] Không [ ] Có	[ ] no [ ] yes	[ ] no [ ] yes	[ ] no [ ] yes
Maximum body temperature *(Thân nhiệt tối đa)*	[__I__].[ ] °C *[ ] Dưới lưỡi [ ] Nách*	[__I__].[ ] °C *[ ] Sublingual [ ] Axillary*	[__I__].[ ] °C *[ ] Sublingual [ ] Axillary*	[__I__].[ ] °C *[ ] Sublingual [ ] Axillary*
Skin or facial flush *(Ửng đỏ da hoặc mặt)*	[ ] Không [ ] Có	[ ] no [ ] yes	[ ] no [ ] yes	[ ] no [ ] yes
Generalized rash *(Hồng ban toàn thân)*	[ ] Không [ ] Có	[ ] no [ ] yes	[ ] no [ ] yes	[ ] no [ ] yes
Abdominal pain *(Đau bụng)*	[ ] Không [ ] Có	[ ] no [ ] yes	[ ] no [ ] yes	[ ] no [ ] yes
Watery stools *(Tiêu lỏng)*	[ ] Không [ ] Có Nếu "Có", số lần [__I__]	[ ] no [ ] yes If 'yes', number [__I__]	[ ] no [ ] yes If 'yes', number [__I__]	[ ] no [ ] yes If 'yes', number [__I__]
Spontaneous bleeding(s) *(Xuất huyết tự nhiên)* **Severity** *(Độ nặng):* Is it clinically significant? *(Có trầm trọng không?)*	[ ] Không [ ] Có **Nếu "Có", mô tả dạng xuất huyết:** [ ] Da [ ] Mũi/nướu răng [ ] Tiêu hóa [ ] Tiết niệu [ ] Âm đạo (bất thường) [ ] Khác: [_____] [ ] Không [ ] Có	[ ] no [ ] yes **If 'yes', specify the type of bleeding:** [ ] Skin [ ] Nose/gum [ ] GI [ ] Urinary tract [ ] Vagina (unusual) [ ] Others: [_____] [ ] no [ ] yes	[ ] no [ ] yes **If 'yes', specify the type of bleeding:** [ ] Skin [ ] Nose/gum [ ] GI [ ] Urinary tract [ ] Vagina (unusual) [ ] Others: [_____] [ ] no [ ] yes	[ ] no [ ] yes **If 'yes', specify the type of bleeding:** [ ] Skin [ ] Nose/gum [ ] GI [ ] Urinary [ ] Vagina (unusual) [ ] Others: [_____] [ ] no [ ] yes
Tourniquet Test: *(performed only if there is no evidence of skin bleeding)* **Dấu dây thắt:** *(chỉ thực hiện nếu không có bằng chứng xuất huyết*	[ ] Không làm [ ] Có làm **Nếu có làm, kết quả:** [ ] âm tính < 20 petechiae [ ] dương tính ≥ 20 petechiae	[ ] not done [ ] done **If done, result:** [ ] negative < 20 petechiae [ ] positive ≥ 20 petechiae	[ ] not done [ ] done **If done, result:** [ ] negative < 20 petechiae [ ] positive ≥ 20 petechiae	[ ] not done [ ] done **If done, result:** [ ] negative < 20 petechiae [ ] positive ≥ 20 petechiae

Clinical Assessment – Cardiovascular: to be filled in at the end of each 24 hour period (each 24 hour period starts at [__I__]/[__I__] hours) **Lâm sàng – Khám tim mạch: điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút)**				
Date of assessment (dd/mm/yy) **Ngày khám (ngày/tháng/năm)**	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
DAY (1 is the enrolment day) Ngày (ngày 1 là ngày bắt đầu nghiên cứu)	**Ngày [ ]**	**Day [ ]**	**Day [ ]**	**Day [ ]**
Edema *(Phù)* **(Code 1=periph.; 2=facial)** *Mã số: 1=ngoại biên; 2=mặt*	[__] Không [__] Có Nếu "Có", mã số [__]	[__] no [__] yes If 'yes', code [__]	[__] no [__] yes If 'yes', code [__]	[__] no [__] yes If 'yes', code [__]
Maximum pulse rate *(Nhịp tim tối đa)*	[__I__I__] /phút	[__I__I__] /min	[__I__I__] /min	[__I__I__] /min
Blood pressure *(Huyết áp)* **Systolic (lowest of the day)** *HA tâm thu (thấp nhất trong ngày)* **Corresponding diastolic** *HA tâm trương tương ứng*	[__I__I__] /mmHg [__I__I__] /mmHg	[__I__I__] /mmHg [__I__I__] /mmHg	[__I__I__] /mmHg [__I__I__] /mmHg	[__I__I__] /mmHg [__I__I__] /mmHg
Pulse pressure ≤ 20 mmHg at any time? *(Hiệu áp ≤ 20 mmHg ở bất kỳ thời điểm nào?)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Clinical shock at any time? *(Có shock không?)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Cyanosis *(Tím tái)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Cold clammy skin *(Da lạnh, ẩm)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Skin mottling *(Da nổi bông)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Capillary refill time >2 sec *(Thời gian hồi phục màu da đầu chi > 2 giây)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Maximum respiratory rate *(Nhịp thở tối đa)*	[__I__] /phút	[__I__] /min	[__I__] /min	[__I__] /min
Respiratory distress *(Suy hô hấp)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Chest pain / discomfort *(Đau ngực)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Abnormal sounds* = Âm bất thường 1 = "sounds like pneumonia" = *"tiếng rales viêm phổi"* 2 = "sounds like pulmonary edema" = *"tiếng rales phù phổi"* 3 = "other" = *"âm thở bất thường khác"*	[__] Không [__] Có Nếu "Có", mã số* [__]	[__] no [__] yes If 'yes', code* [__]	[__] no [__] yes If 'yes', code* [__]	[__] no [__] yes If 'yes', code* [__]
Pleural effusion *Tràn dịch màng phổi (hội chứng 3 giảm)*	[__] Không [__] Có Nếu "Có": [__] P [__] T [__] P+T	[__] no [__] yes If 'yes': [__]R [__]L [__]R+L	[__] no [__] yes If 'yes': [__]R [__]L [__]R+L	[__] no [__] yes If 'yes': [__]R [__]L [__]R+L
Signature of Clinician *Chữ ký của bác sĩ*	[ ]	[ ]	[ ]	[ ]

Clinical Assessment – Abdominal: to be filled in at the end of each 24 hour period (each 24 hour period starts at [__I__]/[__I__] hours) **Lâm sàng – Khám bụng: điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút)**				
Date of Assessment *(dd/mm/yy)***Ngày khám** (ngày/tháng/năm)	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
DAY (1=enrolment day) **Ngày** (ngày 1 là ngày bắt đầu nghiên cứu)	**Ngày [ ]**	**Day [ ]**	**Day [ ]**	**Day [ ]**
Vomiting *(Ói)*	[__] Không [__] Có- Nếu "Có", số lần ói trong 24 giờ là [___]	[__] no [__] yes If 'yes', freq. in 24 hrs [___]	[__] no [__] yes If 'yes', freq. in 24 hrs [___]	[__] no [__] yes If 'yes', freq. in 24 hs [___]
Jaundice *(Vàng da)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Abdominal tenderness *(Đau bụng khi sờ)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Abdominal distension *(Bụng chướng)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Ascites *(Báng bụng – gõ đục vùng thấp)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Liver palpable *(Gan to)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
If 'yes', Liver size *(Nếu "Có", chiều cao gan)*	[__I__] cm dưới bờ sườn phải	[__I__] cm below costal margin	[__I__] cm below costal margin	[__I__] cm below costal margin
Clinical Assessment – Neurological: to be filled in at the end of each 24 hour period (each 24 hour period starts at [__I__]/[__I__] hours) **Lâm sàng – Khám thần kinh: điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút)**				
Convulsion *(Co giật)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Meningism *(Dấu màng não)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Lethargy *(Li bì)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Restlessness *(Bứt rứt)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Glasgow Coma Score* (adults) *Thang điểm Glasgow (người lớn)*	[__]E [__]V [__]M	[__]E [__]V [__]M	[__]E [__]V [__]M	[__]E [__]V [__]M
Blantyre Coma Score (age < 5)** *Thang điểm Blantyre (trẻ < 5 tuổi)*	[__]E [__]V [__]M	[__]E [__]V [__]M	[__]E [__]V [__]M	[__]E [__]V [__]M
Signature of Clinician *Chữ ký của bác sĩ*	[_____]	[_____]	[_____]	[_____]

* **E** Đáp ứng của mắt: 1 = Không đáp ứng; 2 = Mở mắt khi kích thích đau; 3 = Mở mắt theo y lệnh, 4 = Mở mắt tự nhiên

V Đáp ứng về lời nói: 1 = Không đáp ứng, 2 = Phát âm khó hiểu, không rõ chữ, 3 = Nói từng chữ, 4 = Trả lời không đúng; 5 = Trả lời đúng hoàn toàn.

M Đáp ứng về vận động: 1 = Không đáp ứng, 2 = Duỗi cứng mất não, 3 = Gồng cứng mất vỏ, 4 = Đáp ứng đau không chính xác, 5 = Đáp ứng đau chính xác, 6 = Làm đúng theo y lệnh

** **E** Cử động của mắt: 0 = Không nhìn theo, 1 = Nhìn hoặc đưa mắt theo (ví dụ: khuôn mặt của người mẹ)

V Đáp ứng về lời nói: 0 = Không phản ứng khi bị kích thích đau, 1= Rên hoặc phát ra tiếng khóc bất thường khi bị kích thích đau, 2 = Khóc khi bị kích thích đau, hoặc biểu thị bằng lời nói

M Đáp ứng về vận động: 0 = Không phản ứng, hoặc phản ứng không đúng, 1 = Đáp ứng đau không chính xác, 2 = Đáp ứng đau chính xác

Laboratory Tests: to be filled in at the end of each 24 hour period (each 24 hour period starts at [__I__]/[__I__] hours) **Cận lâm sàng: điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút) – (KL = "Không làm")**				
Date of assessment *(dd/mm/yy)* **Ngày thực hiện** (ngày/tháng/năm)	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
DAY (1=enrolment day) Ngày	**Ngày** [ ]	**Ngày** [ ]	**Ngày** [ ]	**Ngày** [ ]
Hematology (the following tests MUST be done at least once a day – xét nghiệm này CẦN làm ít nhất 1 lần trong ngày)				
Haematocrit *(Hct – dung tích hồng cầu)*	Max./Cao nhất [__I__] % Min./Thấp nhất [__I__] %	Max./Cao nhất [__I__] % Min./Thấp nhất [__I__] %	Max./Cao nhất [__I__] % Min./Thấp nhất [__I__] %	Max./Cao nhất [__I__] % Min./Thấp nhất [__I__] %
No. of Hct. Determinations *(Số lần làm Hct)*	[__]	[__]	[__]	[__]
Minimum platelet count *(Số lượng tiểu cầu thấp nhất)*	[__I__I__] x10^9 /L	[__I__I__] x10^9 /L	[__I__I__] x10^9 /L	[__I__I__] x10^9 /L
No. of Plt. Determinations *(Số lần đếm tiểu cầu)*	[__]	[__]	[__]	[__]
Hematology (the following tests MUST be done at enrollment – xét nghiệm này CẦN làm lúc bắt đầu đưa bệnh nhân vào nghiên cứu)				
AST	[__I__I__I__] U/L [__] KL			
ALT	[__I__I__I__] U/L [__] KL			
Minimum total WBC count *(Số lượng bạch cầu thấp nhất)*	[__I__].[__] x10^9/L [__] KL			
Neutrophils *(% Bạch cầu đa nhân)*	[__I__I__] % [__] KL			
Total lymphocytes *(% lympho)*	[__I__I__] % [__] KL			
Hematology (the following tests are optional – xét nghiệm này không bắt buộc)				
AST		[__I__I__I__] U/L [__] KL	[__I__I__I__] U/L [__] KL	[__I__I__I__] U/L [__] KL
ALT		[__I__I__I__] U/L [__] KL	[__I__I__I__] U/L [__] KL	[__I__I__I__] U/L [__] KL
Minimum total WBC count *(Số lượng bạch cầu thấp nhất)*		[__I__].[__] x10^9/L [__] KL	[__I__].[__] x10^9/L [__] KL	[__I__].[__] x10^9/L [__] KL
Neutrophils *(% Bạch cầu đa nhân)*		[__I__I__] % [__] KL	[__I__I__] % [__] KL	[__I__I__] % [__] KL
Total lymphocytes *(% lympho)*		[__I__I__] % [__] KL	[__I__I__] % [__] KL	[__I__I__] % [__] KL
Serum creatinine	[__][__][__] µmol/L [__] KL	[__][__][__] µmol/L [__] KL	[__][__][__] µmol/L [__] KL	[__][__][__] µmol/L [__] KL
Total bilirubin *(Bilirubin toàn phần)*	[__I__].[__] µmol/L	[__I__].[__] µmol/L	[__I__].[__] µmol/L	[__I__].[__] µmol/L
Direct bilirubin *(Bilirubin trực tiếp)*	[__I__].[__] µmol/L	[__I__].[__] µmol/L	[__I__].[__] µmol/L	[__I__].[__] µmol/L

Management (For inpatient only) to be filled in at the end of each 24hour period (each 24 hour period starts at [__I__]/[__I__] hours). **Điều trị (đối với bệnh nhân điều trị nội trú) điền vào cuối mỗi chu kỳ 24 giờ (mỗi chu kỳ 24 giờ bắt đầu lúc [__I__]/[__I__] giờ/phút)**				
Date (dd/mm/yy)	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
DAY (1=enrolment) Ngày	Ngày [___]	day [___]	day [___]	day [___]
Clinical care level *(Cấp độ chăm sóc)**	[___]	[___]	[___]	[___]
Oral intake *(Lượng nước uống vào)*	[__I__I__I__] ml	[__I__I__I__] ml	[__I__I__I__] ml	[__I__I__I__] ml
Parenteral fluid *(Dịch truyền)*	[__] Không [__] Có Nếu "Có": [__I__I__I__] ml	[__] no [__] yes If 'yes': [__I__I__I__] ml	[__] no [__] yes If 'yes': [__I__I__I__] ml	[__] no [__] yes If 'yes': [__I__I__I__] ml
• **For shock resuscitation** *(Để chống sốc)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
• **For rehydration** *(Để bù dịch)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
• **For maintenance ONLY** *(**CHỈ** để duy trì)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Type of fluid *(Loại dịch):* • **Crystalloid** - *Tinh thể (NaCl, LR)* • **Colloid** - *Cao phân tử* • **Blood** - *Máu*	[__] Không [__] Có [__] Không [__] Có Mã số** [___] (nếu có) [__] Không [__] Có Mã số*** [___] (nếu có)	[__] no [__] yes [__] no [__] yes, If yes, Code ** [___] [__] no [__] yes If yes, Code *** [___]	[__] no [__] yes [__] no [__] yes, If yes, Code ** [___] [__] no [__] yes If yes, Code *** [___]	[__] no [__] yes [__] no [__] yes If yes, Code ** [___] [__] no [__] yes If yes, Code *** [___]
• Inotropes (thuốc vận mạch)	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Diuretics *(Lợi tiểu)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Oxygen *(Thở oxy mũi)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Ventilation / resp. support *(Thở máy)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Draining of ascites or pleural effusion? *(dẫn lưu dịch màng phổi, màng bụng?)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes
Dialysis / renal support *(Thẩm phân phúc mạc/chạy thận)*	[__] Không [__] Có	[__] no [__] yes	[__] no [__] yes	[__] no [__] yes

*** Mã số cấp độ chăm sóc:** 1=chăm sóc cơ bản (bệnh nhân tự sinh hoạt được), 2=Can thiệp y tế 1 phần (người thân chăm sóc bệnh nhân theo sự hướng dẫn và hỗ trợ của nhân viêny tế), 3=chăm sóc đặc biệt (do nhân viên y tế chăm sóc). **** Mã số dịch cao phân tử :** 1=Dextrane, 2= Gelatine, 3= Starch, 4=Albumin; 5=kết hợp nhiều loại dịch; ***** Mã số máu:** 1=máu toàn phần; 2=hồng cầu lắng, 3=huyết tương tươi đông lạnh; 4=tiểu cầu đậm đặc; 5=kết tủa lạnh (các yếu tố đông máu); 6= kết hợp nhiều loại chế phẩm máu

Ultrasound to be performed within 24h of defervescence (Siêu âm được làm 24 giờ sau khi hết sốt)		
Ngày thực hiện ***(ngày/tháng/năm)***	[__I__]/[__I__]/[__I__]	[__I__]/[__I__]/[__I__]
Ngày (ngày 1 là ngày bắt đầu nghiên cứu)	**Ngày [___]**	**Ngày [___]**
Ultrasound *(Siêu âm bụng)*	Các điều kiện làm siêu âm [__] Tốt [__] Khá [__] Xấu	Các điều kiện làm siêu âm [__] Tốt [__] Khá [__] Xấu
	Ascites [__] Không [__] Có *(Báng bụng)*	Ascites [__] Không [__] Có *(Báng bụng)*
	Pleural effusion [__] Không [__] Có *(Dịch màng phổi)*	Pleural effusion [__] Không [__] Có *(Dịch màng phổi)*
	Pericardial effusion [__] Không [__] Có *(Dịch màng tim)*	Pericardial effusion [__] Không [__] Có *(Dịch màng tim)*
	Thickening of gall bladder [__] Không [__] Có *(Dày vách túi mật)*	Thickening of gall bladder [__] Không [__] Có *(Dày vách túi mật)*
	Peri-gall bladder fluid [__] Không [__] Có *(Tụ dịch quanh túi mật)*	Peri-gall bladder fluid [__] Không [__] Có *(Tụ dịch quanh túi mật)*
	Peri-renal fluid mass [__] Không [__] Có *(Tụ dịch quanh thận)*	Peri-renal fluid mass [__] Không [__] Có *(Tụ dịch quanh thận)*
	Enlargement of liver [__] Không [__] Có *(Gan to)*	Enlargement of liver [__] Không [__] Có *(Gan to)*

This section to be completed by the Principal Investigator only		
This is a case of:		**Essential Criteria (Fever plus X)**
Probable Dengue fever	[ ] Không [ ] Có	Two or more of the following: fever, headache, retro-orbital pain, myalgia arthralgia, rash, haemorrhagic manifestations, leukopaenia
DHF Grade I	[ ] Không [ ] Có	• Thrombocytopaenia (<100,000/mm^3) • Evidence of **induced, but not spontaneous**, haemorrhagic manifestations • Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings e.g. ascites
DHF Grade II	[ ] Không [ ] Có	• Thrombocytopaenia (<100,000/mm^3) • Evidence of **spontaneous** haemorrhagic manifestations • Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values ≥20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings
DHF Grade III	[ ] Không [ ] Có	• Thrombocytopaenia (<100,000/mm^3) • Evidence of spontaneous haemorrhagic manifestations • Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values >20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings • Narrowed pulse pressure ≤20 mm/Hg • Clinical signs of circulatory shock, e.g. refill time >2 secs, cold, clammy skin, cyanosis • Rapid pulse
DHF Grade IV	[ ] Không [ ] Có	• Thrombocytopaenia (<100,000/mm^3) • Evidence of spontaneous haemorrhagic manifestations • Evidence of capillary leakage, e.g. >20% increase in Hct during hospitalization, OR Hct values >20% of the normal value for age and sex, OR >20% decrease after i.v. fluid infusion OR pleural effusions, ascites OR ultrasound findings e.g. ascites • Pulse pressure=0 or undetectable blood pressure • Clinical signs of circulatory shock, e.g. refill time >2 secs, cold, clammy skin, cyanosis • Rapid pulse
Cannot classify	[ ] Không [ ] Có	

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